Effect of cadmium on anion exchange capability through Band 3 protein in human erythrocytes

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Abstract

The efficiency of transport through Band 3 protein, mediating HCO_3^-/Cl^- exchange across erythrocytes membrane, is reduced by oxidative stress. The aim of the present study was to verify whether Band 3 protein transport efficiency is compromised by treatment with Cadmium (Cd^{2+}), an extremely toxic heavy metal known to interfere with antioxidant enzymes, energy metabolism, gene expression and cell membranes. To this end, the rate constant for $SO_4^=$ uptake through Band 3 protein (accounting for velocity of anion exchange) was measured along with membrane –SH groups, Malonyldialdehyde (MDA) and Band 3 protein expression levels in Cd^{2+}-treated human erythrocytes (300 µM, 1 mM). Our results show that Cd^{2+} reduced the rate constant for $SO_4^=$ uptake, with a significant increase in MDA levels at both concentrations and with a reduction in –SH groups observed after 1 mM Cd^{2+} treatment, whereas Band 3 protein expression levels were unchanged in both conditions. In conclusion: i) Cd^{2+} reduces Band 3 protein transport efficiency *via* different mechanisms depending on metal concentration and with unchanged expression levels; ii) the assessment of Band 3 protein anion exchange capability is a good tool to assay the impact of heavy metals on cell homeostasis and, possibly, useful for diagnosis and monitoring of development of Cd^{2+} toxicity-related pathologies.

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Introduction

Band 3 protein is the most abundant integral protein of erythrocyte membrane[1] and its crystal structure was defined in 2015.[2] Band 3 protein has been extensively studied in wild-type human mature erythrocytes, during their maturation, and in connection with possible defects of membrane skeleton.[3,4] It is critically involved in maintenance of erythrocytes deformability and ion balance essential to gas exchange efficiency, making thus the evaluation of anion exchange capability (Cl^-/HCO_3^-) useful to check erythrocytes homeostasis in health and disease.[5,6] Band 3 protein functions are mediated by two domains, a membrane domain for anion exchange and a cytoplasmic domain which mainly contributes to the protein-protein interactions, by coupling the lipid bilayer to the underlying cytoskeleton, through cysteine –SH groups.[7] Human erythrocytes, being constantly exposed to oxidative stress, have been already used as a model to study possible alterations due to oxidants like H_2O_2 or *N*-Ethylmaleimide (NEM) at level of methemoglobin production, lipid peroxidation, membrane –SH groups oxidation, efficiency of anion exchange capability through Band 3 protein and phosphorylation of Band 3 protein Tyrosine residues.[8,9]

Among oxidants, Cadmium (Cd^{2+}), a widely distributed and extremely toxic heavy metal, has been proven to alter antioxidant enzymes, energy metabolism, membrane arrangement, gene expression and apoptosis, though the exact mechanism of its toxicity is still not completely understood.[10–13] It enters blood stream, binds to the erythrocyte membrane and stimulates reactive oxygen species (ROS) formation,[10] affecting the antioxidant system and increasing lipid peroxidation.[10,13] In addition, Cd^{2+} has been shown to alter lipid bilayer molecular structure, thus modifying the biophysical properties of cell membrane such as fluidity, which in turn affects signals transduction, channel functions and protein activity.[14]

Cadmium is found in many natural foods, particularly in grains and seaweed among vegetables, and, with regard to animals, in fishes and shellfishes. As pointed out by Satarug *et al.*,[15] human exposure to Cd^{2+} is mostly through foods (90%) and has been

linked to increased risk of bone fracture, cancer, kidney dysfunction, and hypertension.[15] However, cigarette smoke should be not excluded as source of the body cadmium Cd^{2+} burden, being tobacco plant a cadmium bioaccumulator.[16] In any case, once absorbed into blood circulation, Cd^{2+} may exert its toxic effect by damaging cell membranes[17] at concentrations 10 folds lower than those currently used in *in vitro* assays.[14] Despite this discrepancy, *in vitro* experiments have been conducted in order to rapidly evaluate Cd^{2+} effects at cellular level after brief exposure to the metal.[14,18] In particular, Suwalski *et al.*[14] point out that 1 mM Cd^{2+}, from one hand is effective in altering erythrocytes membrane, and, from the other hand, similarly to other *in vitro* studies,[17,19] is at least 10-folds higher than its toxic concentration in blood.[20]

On these premises, the present investigation aims to verify whether and how Band 3 protein transport efficiency in human erythrocytes is affected by a transient exposure to Cd^{2+} and whether monitoring anion exchange capability may be considered as an additional tool for diagnosis and monitoring of development of pathologies related to cadmium Cd^{2+} toxicity.

To this end, erythrocytes have been exposed for 1 h at Cd^{2+} (300 µM and 1 mM), in order to produce oxidative damage, according to other *in vitro* investigations,[14] assaying the effects of Cd^{2+} within this range of concentrations.

Cd^{2+}-induced oxidative damage has been assessed by measuring levels of both membrane –SH groups, mostly deriving from Band 3 protein,[21] and Malonyldialdehyde (MDA) – the end product of lipid peroxidation – while the efficiency of anion transport through Band 3 protein has been monitored by determining the rate constant for $SO_4^=$ uptake, which accounts for exchange velocity and is more easily estimated than Cl^- or HCO_3^- uptake.[22–24] Expression levels of Band 3 protein in Cd^{2+}-treated erythrocytes have been also determined.

Materials and Methods

Erythrocytes preparation

Human blood was obtained from healthy volunteers upon informed consent. Blood was collected in heparinized tubes, washed in an isotonic solution (composition in mM: 145 NaCl, 20 HEPES (4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid), pH 7.4, osmotic pressure 300 mOsm) and centrifuged thrice (ThermoScientific, 1000 g, 5 min) to remove plasma and buffy coat.

$SO_4^=$ uptake measurement

Human erythrocytes were suspended to 3% hematocrit in 35 mL isotonic $SO_4^=$-containing medium defined as $SO_4^=$ medium (composition in mM: 118 Na_2SO_4, 20 HEPES, 15 glucose, pH 7.4, osmotic pressure 300 mOsm). At specified time intervals (5-10-15-30-45-60-90-120 min), 5 mL samples of erythrocytes suspension were treated with 10 µM 4,4'-diisothiocyanatostilbene-2, 2'-disulphonic acid (DIDS), a specific and irreversible blocker of Band 3 protein[25] and kept on ice. After the last sample withdrawal, erythrocytes were washed thrice by centrifugation in cold isotonic solution (ThermoScientific, 4°C, 1000 g, 5 min) to remove $SO_4^=$ from the external medium and then hemolysed by 1 mL distilled water, while proteins were hydrolysed with 4% v/v perchloric acid. Centrifugation (4°C, 2400 g, 10 min) allowed to obtain $SO_4^=$-containing supernatant used for turbidimetric method. $SO_4^=$ was precipitated by adding 500 µL supernatant from each sample to 1 mL glycerol and distilled water solution (1:1), 1 mL 4 M NaCl plus hydrochloric acid (HCl 37%) solution (12:1) and 500 µL 1.24 M $BaCl_2 \cdot 2\,H_2O$. Total amount of $SO_4^=$ internalized by erythrocytes at fixed times was spectrophotometrically quantified (425 nm wavelength, Beckman DU 640) and the absorption converted to mM of $SO_4^=$ using a calibrated standard curve previously obtained by precipitating known $SO_4^=$ amounts. The rate constant, measured in min^{-1}, was then calculated by the following equation: $C_t = C_\infty (1^{-e-rt}) + C_0$, where C_t, C_∞ and C_0 represent the intracellular $SO_4^=$ total amount measured at time t, 0 and ∞ respectively; *e* indicates Neper number (2.7182818); *r* is the rate constant of the transport process and *t* is time fixed for each sample withdrawal (5-10-15-30-45-60-90-120 min). The reciprocal of rate constant for $SO_4^=$ uptake (min) represents the time needed to reach 63% of total $SO_4^=$ intracellular total amount.[23]

With regard to Cd^{2+} treatment, erythrocytes, after washing, were diluted to 3% hematocrit and treated with $CdCl_2$ doses comprised between 50 µM and 1 mM for one hour at 37°C in isotonic solution. During incubation, light microscope observations were performed to exclude possible Cd^{2+}-dependent hemolysis. After incubation, samples were centrifuged (1000 g, 5 min) to remove the supernatant, re-suspended to 3% hematocrit in $SO_4^=$ medium containing Cd^{2+} and $SO_4^=$ uptake measured as described for control conditions.

Based on $SO_4^=$ uptake measurement, two Cd^{2+} doses (300 µM and 1 mM) have been chosen to complete the experimental design.

Membrane –SH groups determination

Membrane –SH groups estimation was performed on erythrocytes (untreated or treated with Cd^{2+}) according to Roy and co-workers.[21] In details, erythrocytes, after washing, were treated with 300 µM and 1 mM Cd^{2+} to 3% hematocrit for one hour at 37°C. After the incubation, they were centrifuged (ThermoScientific, 1000 g, 5 min), concentrated to 10% hematocrit and lysed by cold hypotonic buffer (2.5 mM NaH_2PO_4, 5 mM HEPES). After 10 min stirring at 0°C, hemoglobin and intracellular content were discarded by repeated centrifugations (Eppendorf microfuge, 4°C, 18000 g, 20 min). The process was repeated with the same hypotonic buffer to discard hemoglobin. One volume of membranes (from both treated and untreated erythrocytes) was then incubated with nine volumes of 0.1 M NaOH for 30 min at 0°C plus 200 µM dithiothreitol and 20 µg/ml Phenylmethylsulfonil fluoride (PMSF). After incubation, samples were centrifuged (4°C, 18000 g, 45 min). The pellet, containing Band 3 protein, was washed thrice with 5 mM sodium phosphate (pH 8.0) and used for –SH groups determination. For this purpose, pellet (200 µl) was solubilized by incubating 300 µL of 20% v/v Sodium dodecyl sulphate (SDS) reagent in 3 ml of 100 mM sodium phosphate (pH 8.0), for 30 min at 37°C. Samples were further incubated with 100 µl of 10 mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) in 100 mM sodium phosphate (pH 8.0), for 20 min at 37°C. DTNB reacts specifically with thiol groups producing a highly colored yellow anion. Levels of membrane –SH groups were spectrophotometrically read at 405 nm[21] and expressed as percentage of –SH groups in untreated erythrocytes.

Determination of Malondialdehyde levels

To assess oxidative stress, levels of MDA, as end product of lipid peroxidation, have been measured on both treated and untreated erythrocytes.[26] The assay is based on the reaction between MDA and thiobarbituric acid (TBA) which produces thiobarbituric acid-reactant substances, colorimetrically detectable at 532 nm wavelength. Erythrocytes, after washing, were suspended to 3% hematocrit in isotonic medium and incubated with either 300 µM or 1 mM $CdCl_2$ plus 2 mM NaF for one hour at 37°C. Samples were

centrifuged (ThermoScientific, 1000 g, 5 min), suspended to 10% hematocrit in 1 mL distilled water to induce hemolysis and frozen overnight at –20°C until analysis. After thawing, sample aliquots (200 µL) were treated with 500 µL TBA (1% v/v dissolved in 1 N HCl) and incubated at 95°C for 1 h. Samples were then cooled on ice, centrifuged (13000 g, 15 min, 4°C) and the supernatant spectrophotometrically read at 532 nm. MDA levels were converted in micromolar by comparing results with a calibration standard curve previously obtained by known concentrations of 1,1,3,3-tetramethoxypropan 99% MDA bis (dimethyl acetal) 99% (Sigma).

Erythrocytes membrane preparation and Sodium dodecyl sulphate-PolyAcrylamide gel electrophoresis

Membrane extracts were prepared as previously described[9] with slight modifications. Briefly, after washing, packed erythrocytes were diluted into 1.5 mL of cold hemolysis buffer (2.5 mM Sodium Phosphate, 1 mM EGTA, pH 8) containing a protease and phosphatase inhibitor cocktail (1 mM PMSF, 1mM NaF, 1 mM Sodium Orthovanadate) and then repeatedly centrifuged (Eppendorf, 13000 g, 4°C) to discard hemoglobin. Membrane were then solubilized by 1% (v/v) SDS and incubated on ice for 20 min. Solubilized membrane proteins, contained in the supernatant, were addressed to protein content quantification, according to Bradford method[27] and frozen at −80°C until use. Membranes obtained from each experimental condition, once thawed, were solubilized in Laemmli Buffer[28] in a volume ratio of 1:1, heated for 5 min at 95°C and then loaded (2 µg proteins). Samples were then separated on 12% polyacrylamide gel under reducing conditions and then transferred to polyvinylidenefluoride (PVDF) membrane.

Western blot analysis

PVDF membranes were incubated at 4°C overnight with monoclonal anti-Band 3 protein (1:100000; Santa Cruz Biotechnology, produced in mouse) diluted in 1×phosphate-buffered saline, 5% (w/v) non-fat dried milk and 0.1% Tween-20. Membranes were then incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:5000, Affini Pure), for 1 h at room temperature, followed by chemioluminescent detection, according to the manufacturer's instruc-tions (Super Signal West Pico Chemiluminescent Substrate, Pierce Thermo Scientific, Rockford, IL, USA). To verify whether blots contained equal amounts of protein, they were also incubated with monoclonal antibodies against β-actin (1:1000, Santa Cruz Biotechnology) produced in mouse. Quantification of Band 3 protein (approximately 95 kDa) expression was done by densitometry (Bio-Rad ChemiDoc™ XRS equipped with Image Quant 171 TL, v2003) and standardized to β-actin levels. Molecular weight standards, ranging between 10 and 250 kDa, were used to define molecular weight positions.

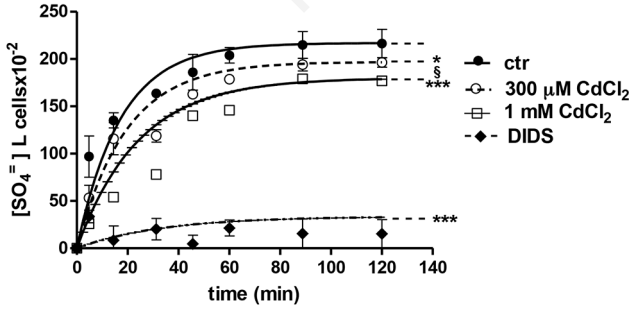

Figure 1. Time course of $SO_4^=$ uptake measured in control (untreated erythrocytes) or in erythrocytes treated with either 300 µM Cd^{2+}, or 1 mM Cd^{2+} or 10 µM 4,4'-diisothiocyanatostilbene-2, 2'-disulphonic acid (DIDS). *P<0.05 and ***P<0.001 *vs* control, §P<0.05 *vs* 300 µM Cd^{2+}, as determined by one-way analysis of variance followed by Bonferroni's Multiple Comparison *post hoc* test, by comparing all values of theoretical curves, at all time points (N=6).

Experimental data and statistics

Data are expressed as means ± S.E.M. GraphPad Prism software (version 5.00 for Windows; San Diego, CA) was used. Significant differences between means were tested by paired one-way analysis of variance (ANOVA), followed by Bonferroni's, *post hoc* test. Statistically significant differences were assumed at P<0.05 (*P<0.05, **P<0.01, ***P<0.001); *N* represents the number of independent experiments.

Results

$SO_4^=$ uptake measurement

Treatment with Cd^{2+} at all concentrations did induce hemolytic events. With regard to $SO_4^=$ uptake measurement, doses lower than 300 µM did not significantly alter the rate constant, therefore data presented in Figure 1 refer to $SO_4^=$ uptake in either 300 µM or 1 mM Cd^{2+}-treated erythrocytes, compared to control (untreated erythrocytes) and reported as a function of time. The velocity of this process is represented by the rate constant for $SO_4^=$ uptake (min^{-1}).

$SO_4^=$ transport in control erythrocytes progressively increased and reached equilibrium in 30 min, with a rate constant of 0.057±0.001 min^{-1} (time to reach 63% of total $SO_4^=$ intracellular total amount=17 min). Erythrocytes treated with 300 µM Cd^{2+} exhibited a rate constant of 0.051±0.001 min^{-1} (19 min), significantly lower than those observed in control conditions (P<0.05) and total amount of $SO_4^=$ internalized at 30 min and 45 min by such treated cells (118.75±6.5 mM and 162.72±4.3 mM respectively, Table 1) was significantly lower those determined in control at both time intervals (163.72±2.4 mM and 186±18 mM respectively, Table 1). Similarly, erythrocytes exposed to 1 mM Cd^{2+} showed a rate constant for $SO_4^=$ uptake (0.041±0.001 min^{-1}, 24 min) significantly lower than control (P<0.001) and $SO_4^=$ total amount trapped by the cells at 30 min and 45 min (78.52±11 mM and 140±8.8 mM respectively, Table 1) significantly lower than what determined in untreated erythrocytes at both time intervals (163.72±2.4 mM and 186±18 mM respectively, Table 1).

Treatment with 10 µM DIDS applied at the beginning of incubation in $SO_4^=$ medium completely blocked $SO_4^=$ uptake (rate constant of 0.018±0.001 min^{-1}, 55 min) and significantly reduced $SO_4^=$ total amount detected at both 30 and 45 min (20.25±12 mM and 4.75±9 mM respectively, Table 1) with respect to both control and Cd^{2+}-treated cells (P<0.001).

As said, Cd^{2+} concentrations comprised between 50 and 200 µM did not alter the rate constant for $SO_4^=$ uptake when compared to the control, and, hence, have been not shown and no longer considered for the experimental protocol.

Membrane –SH groups determination

Determination of membrane –SH groups was performed in erythrocytes treated with either 300 µM or 1 mM Cd^{2+} (Figure 2). After exposure to 300 µM Cd^{2+}, levels of membrane –SH groups

were not significantly different when compared to control (untreated erythrocytes), while, after treatment with 1 mM Cd^{2+}, significantly lower than those of untreated erythrocytes (P<0.001). Results were compared to what obtained after treatment with 2 mM NEM, a thiol oxidizing compound, which significantly reduced –SH groups when compared to control (Figure 2, P<0.001).

Malondialdehyde membrane levels

As shown in Figure 3, levels of MDA, the end product of lipid peroxidation, in erythrocytes treated with either 300 µM or 1 mM Cd^{2+} were significantly higher than those measured in control conditions (untreated erythrocytes, P<0.001). These data have been compared with MDA levels produced by the oxidant NaF, known to induce a significant lipoperoxidation. As expected, in this latter case MDA levels were significantly higher than those measured in both control and Cd^{2+} treated (300 µM and 1 mM) erythrocytes (P<0.001).

Western blot analysis

Band 3 protein expression levels in erythrocytes treated with either 300 µM or 1 mM Cd^{2+} were not significantly different with respect to those determined in untreated erythrocytes (Figure 4).

Discussion

In the present investigation experiments have been carried out to evaluate anion exchange capability through Band 3 protein after a brief exposure of human erythrocytes to Cd^{2+}. For this purpose, the rate constant for $SO_4^=$ uptake has been determined, as $SO_4^=$ can be more slowly exchanged than Cl^- and, hence, more easily measured.[2,23] This parameter accounts for one of Band 3 protein functions, which, as currently known, involves gas exchange, membrane deformability and ion balance across erythrocytes

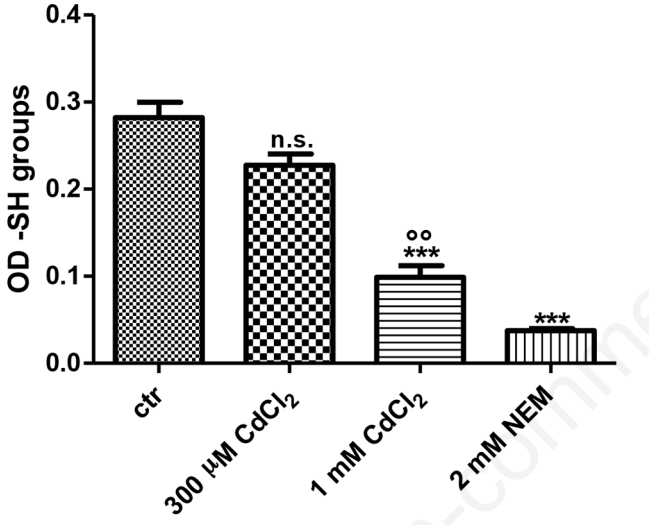

Figure 2. Membrane –SH groups levels (expressed as optical density, OD measured in control (untreated erythrocytes) or in either 300 µM or 1 mM Cd^{2+} or 2 mM *N*-Ethylmaleimide (NEM)-treated erythrocytes. Bars represent the mean ± SEM from at least 8 experiments, where n.s. not significant *vs* control, **P<0.001 *vs* control,°°P<0.01 *vs* 300 µM Cd^{2+}, §§§P<0.001 *vs* 300 µM Cd^{2+}, #P<0.05 *vs* 1 mM Cd^{2+}, as determined by one way analysis of variance followed by Bonferroni's Multiple Comparison *post hoc* test.

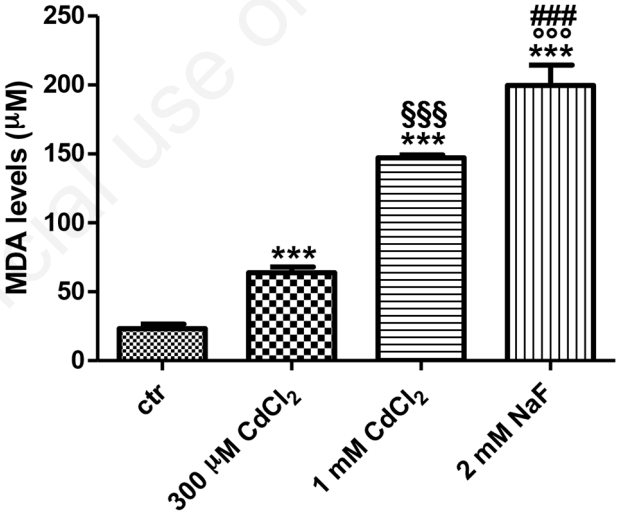

Figure 3. Malonyldialdehyde (MDA) levels (expressed as µM) measured in control (untreated erythrocytes) and in erythrocytes treated with either 300 µM or 1 mM Cd^{2+} or 2 mM NaF. Bars represent the mean ± SEM from at least 7 experiments, where ***P<0.001 *vs* control, §§§P<0.001 *vs* 300 µM Cd^{2+},°°°P<0.001 *vs* 300 µM Cd^{2+}, ###P<0.001 *vs* 1 mM Cd^{2+} as determined by one way analysis of variance followed by Bonferroni's Multiple Comparison *post hoc* test.

Table 1. Amount of $SO_4^=$ trapped in human erythrocytes during $SO_4^=$ kinetics experiments and measured at both 30 and 45 min (at equilibrium) in control conditions (untreated erythrocytes) or in erythrocytes treated with either $CdCl_2$ at different concentrations (300 µM and 1 mM) or 10 µM 4,4'-diisothiocyanatostilbene-2, 2'-disulphonic acid (DIDS). Data are presented as means ± SEM from separate *N* experiments, where: ***P<0.001 and **P<0.01 vs control; §P<0.05 *vs* 300 µM CdCl2 as determined by one-way analysis of variance followed by Bonferroni's Multiple Comparison *post hoc* test.

Time (min)	mM $SO_4^=$ trapped by erythrocytes				N
	Control	300 µM $CdCl_2$	1 mM $CdCl_2$	10 µM DIDS	
30 min	163.72±2.4	118.75±6.5**	78.52±11***,§	20.25±12***	4
45 min	186.00±18	162.72±4.3**	140.00±8.8***,§	4.75±9***	4

membrane.[2,23] The efficiency of Band 3 protein in anion exchange not only depends on an intact protein structure, but also on its crosslink with other proteins, including cytoskeletal components, hemoglobin and Glucose-6-phosphate dehydrogenase, placed underneath erythrocytes membrane.[29] Hence, alterations at level of Band 3 protein may impact on these components, and *vice versa*.

In the recent years, monitoring of Band 3 protein anion exchange capability has been more widely used to assay the effect of different conditions, *i.e.* toxins and oxidants[6,22, 30–32] or of diseases.[5,6] In this context, as previous investigations revealed that the rate constant for $SO_4^=$ uptake is a sensitive tool to detect the effects of xenobiotics,[22] this parameter has been used in the present work to verify the effect of Cd^{2+} on erythrocytes homeostasis, in an attempt to provide a novel tool for diagnosis and monitoring of development of cadmium Cd^{2+} toxicity-related pathologies. This issue is in line with what recently proven by Morabito *et al*., reporting about Band 3 protein monitoring in diseases associated to oxidative stress, such as canine leishmaniasis[33] and Systemic sclerosis.[5]

The present results show that the rate constant for $SO_4^=$ uptake is significantly reduced by a transient exposure to both 300 µM and 1 mM Cd^{2+}, putatively mediated by a significant increase in MDA levels in both experimental conditions and a decrease in membrane –SH groups only in 1 mM Cd^{2+} -treated erythrocytes. Interestingly, the metal seems not to alter the expression levels of Band 3 protein.

At his point, we may suggest that the reduction in anion exchange capability is affected by Cd^{2+} *via* different mechanisms depending on Cd^{2+} concentration, as the oxidative state of membrane –SH groups is not affected by low-concentrated Cd^{2+}. Hence, an impairment of Band 3 protein function in this experimental condition may be more likely due to a lipoperoxidative effect of Cd^{2+}, as attested by high MDA levels, rather than on a direct oxidation of membrane –SH groups. Though this result is in line with what already described by Tezcan *et al*.,[18] reporting about MDA production in human erythrocytes after 1 hour Cd^{2+} treatment, its novelty relies in the altered anion exchange capability through Band 3 protein, never proven so far.

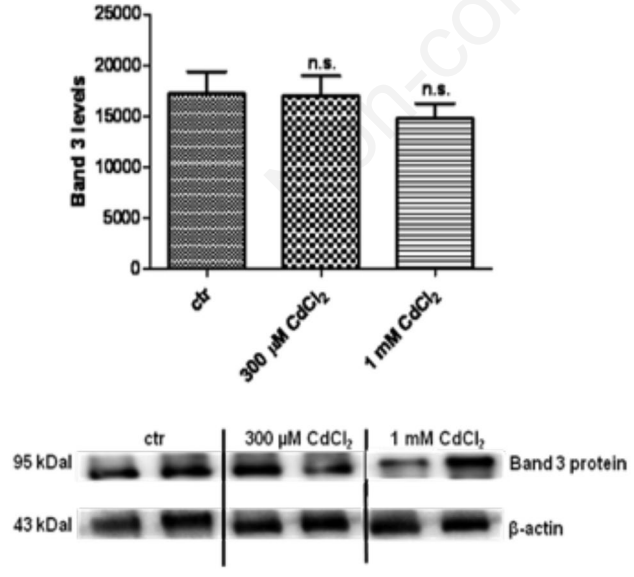

Figure 4. Band 3 protein and β-actin expression levels in control (untreated erythrocytes) and in erythrocytes treated with either 300 µM or 1 mM Cd^{2+} detected by Western blot analysis. N.s. not significant *vs* control as determined by one-way analysis of variance followed by Bonferroni's Multiple Comparison *post hoc* test (N=4).

The evidence that oxidation state of membrane –SH groups is critically involved in the efficiency of transport through Band 3 protein is actually supported by other studies, as –SH groups mostly belong to Band 3 protein structure.[21] The importance of changes in Band 3 protein structure caused by cysteine –SH groups oxidation, especially after exposure to NEM and pH 6.5, has been already proven.[34] Nevertheless, the correspondence between reduced membrane –SH groups and Band 3 protein anion exchange capability may be not always univocal, as Morabito *et al.*,[30] have demonstrated that H_2O_2, used as an oxidant molecule on human erythrocytes, significantly reduced the rate constant for $SO_4^=$ uptake through Band 3 protein, but did not alter –SH groups levels. At this point, we could even suggest that 300 µM Cd^{2+} may act on Band 3 protein with a mechanism similar to that one exerted by H_2O_2[30] with the difference that, based on the present findings, Cd^{2+} affects Band 3 protein *via* lipid peroxidation, while H_2O_2, not inducing lipid peroxidation, seems to inflict damage by putatively affecting cytoplasmic components, cross linking with Band 3 protein.[30]

As said, a second result from the present study, confirming what already reported by other authors,[18,35] is that Cd^{2+} induces oxidative stress by increasing lipid peroxidation. MDA is a marker of membrane lipid peroxidation resulting from the interaction of ROS and cell membrane, which may be attributed to alterations in the antioxidant defense system, including glutathione (GSH) peroxidase, superoxide dismutase, catalase as well as non-enzymatic antioxidants, such as reduced glutathione.[19,36] In particular, cadmium Cd^{2+} has high affinity for cell membrane, probably deriving from its strong interaction with lipids.[37] Both 300 µM and 1 mM Cd^{2+} CdCl2 , which did not induce hemolysis, induced lipid peroxidation on erythrocytes membrane, with consequent impairment of anion exchange capability through Band 3 protein. A cell membrane re-arrangement due to Cd^{2+}-induced lipid peroxidation, in line with what previously observed by Suwalsky *et al*.,[14] seems to be responsible for the reduction in rate constant for $SO_4^=$ uptake observed after exposure to 300 µM Cd^{2+}. Noteworthy, the hypothesis that Cd^{2+}-induced lipoperoxidation may contribute to the membrane –SH groups decrease observed after treatment with 1 mM Cd^{2+} CdCl2 can't be excluded at all. In this regard, other authors also showed that oxidative events from alcohol administration clearly induced erythrocytes lipid peroxidation increase, –SH groups decrease and deplation of antioxidant enzyme activities.[38] On this basis lipid peroxidation would affect not only the arrangement of membrane bilayer, which *per se* is sufficient to reduce the rate constant of $SO_4^=$ uptake,[5,33] but also the oxidation state of membrane –SH groups, essential for Band 3 protein function. Such combined effect would finally result in a reduction in anion exchange capability.

To better focus on the effect of Cd^{2+}, expression levels of Band 3 protein in treated erythrocytes have been evaluated. As the decrease in the rate constant for $SO_4^=$ uptake observed in Cd^{2+}-treated erythrocytes is not associated to reduced Band 3 protein expression levels, the inhibitory effect would be rather due to the oxidation state of Band 3 protein –SH groups, or lipoperoxoidative events when high concentrated Cd^{2+} is considered. Therefore, these findings corroborate the hypothesis that the rate constant for $SO_4^=$ uptake is a sensible tool to evaluate erythrocytes homeostasis under oxidative conditions, namely when changes in parameters currently associated to oxidative stress are undetectable. Moreover, Band 3 protein transport efficiency may be useful to assay the effects of heavy metals, whose impact on public health represents a common threat.

One more finding arising from the present work is related to Cd^{2+} concentrations. In particular, to prove a possible effect of Cd^{2+} on Band 3 protein, a brief exposure to the metal, according to what reported in other *in vitro* tests[14] has been used. As pointed out by Suwalsky *et al.,*[14] Cd^{2+} was applied at concentrations up to 10 folds higher than those either used in *in vivo* experiments or reported as tolerable weekly intake in humans, when Cd^{2+} enters food chain.[18] Nonetheless, they are not hemolytic and allow to rapidly evaluate erythrocytes function and morphology after a brief exposure to the metal.

As Cd^{2+} concentrations lower than 300 µM are proven not to alter the rate constant for $SO_4^=$ uptake (data not shown), they could be proposed as not observed effect levels, though an *in vivo* investigation is required to better prove this aspect.

Among heavy metals, cadmium Cd^{2+} displays a variety of effects[35,39,40] and molecular mechanisms have been tentatively explained, though not completely unraveled. The evidence that membrane transport systems, along with antioxidants enzymes activity and GSH levels are affected in *in vivo* models by exposure to Cd^{2+} has been already proven,[13] but such effects have been described after chronic Cd^{2+} administration, which implies that detoxification systems and time to reach the Minimum Effective Dose should be taken into account. In this context, the present investigation would add some more knowledge about the impact of this metal on cell membrane and ion transport systems. Morabito and co-workers[41] have already demonstrated that HEK 293 Phoenix cells, exposed to 200 µM Cd^{2+} are not able to exhibit a homeostatic response to hyposmotic shock, referred to as Regulatory Volume Decrease, common to many cell types and mediated by both ion transport through channels and exchangers.[42] The evidence that ion transport can be targeted by Cd^{2+} motivated the present choice of verifying the efficiency of a specific ion exchanger, such as Band 3 protein, in an anucleated cell, based also on Tezcan *et al*.,[18] hypothesis for explaining Cd^{2+} mechanism of action. These authors point out that a direct interaction of Cd^{2+} with ion transport systems may explain Cd^{2+} toxicity, though an inter-action between the metal and membrane phospholipids affecting ion channel activity can't be excluded at all. On this basis, Cd^{2+} toxicity is not specific at the membrane level.

Erythrocytes have been reasonably chosen as a model for this type of investigation, being continuously threatened by oxidative events, namely related to aging, strenuous exercise, and pathologies associated to high ROS levels.[43,44] Therefore, their possible adaptation to oxidative stress, and in particular to Cd^{2+}-induced oxidative stress, could add more information to both toxicology and oxidative stress impact on cells.

Conclusions

Taken together these findings show that: i) a significant reduction in the rate constant for $SO_4^=$ uptake through Band 3 protein is seen after exposure to Cd^{2+}; ii) such reduction is mainly due to lipid peroxidation at low Cd^{2+} concentrations, while, at higher concentrations, to a possible combined effect lipid peroxidation acting on lipid bilayer arrangement and on membrane –SH groups oxidation as well; iii) in both cases expression levels of Band 3 protein are not altered; iv) Band 3 protein anion exchange capability is a suitable tool to monitor toxic effects of Cd^{2+}. Further studies are needed to better focus on signaling pathways possibly involved in alterations of Band 3 protein function with possible use of antioxidants preventing detrimental Cd^{2+} effects.

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