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Hexavalent chromium-induced erythrocyte membrane phospholipid asymmetry

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Abstract Hexavalent (VI) chromium is a global contaminant with cytotoxic activity. Chromium (VI) induces oxidative stress, inflammation, cell proliferation, malignant transformation and may trigger carcinogenesis and at the same time apoptosis. The toxic effects of chromium (VI) at least partially result from mitochondrial injury and DNA damage. Erythrocytes lack mitochondria and nuclei but may experience an apoptosis-like suicidal cell death, i.e. eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Eryptosis may result from increase of cytosolic Ca^{2+} activity, ATP depletion and/or ceramide formation. The present study explored, whether chromium (VI) triggers eryptosis. Fluo-3-fluorescence was employed to determine cytosolic Ca^{2+}-concentration, forward scatter to estimate cell volume, binding of fluorescent annexin V to detect phosphatidylserine exposure, hemoglobin concentration in the supernatant to quantify hemolysis, luciferin–luciferase to determine cytosolic ATP concentration and fluorescent anti-ceramide antibodies to uncover ceramide formation. A 48 h exposure to chromium (VI) ($\geq$10 μM) significantly increased cytosolic Ca^{2+}-concentration, decreased ATP concentration (20 μM), decreased forward scatter, increased annexin V-binding and increased (albeit to a much smaller extent) hemolysis. Chromium (VI) did not significantly modify ceramide formation. The effect of 20 μM chromium (VI) on annexin V binding was partially reversed in the nominal absence of Ca^{2+}. The present observations disclose a novel effect of chromium (VI), i.e. Ca^{2+} entry and cytosolic ATP depletion in erythrocytes, effects resulting in eryptosis with cell shrinkage and cell membrane scrambling.

Keywords Phosphatidylserine · Chromium (VI) · Calcium · Cell volume · Eryptosis

Introduction

Industrialization resulted in the global occurrence of soluble hexavalent (VI) chromium (Ramirez-Diaz et al. 2008; Velma et al. 2009), which is a component of wood preservatives, pigments, anticorrosive primers, metal plating, fossil fuel, etc. (Thompson et al. 2011). In some areas, chromium (VI) contaminates the ground water (Oze et al. 2007). Chromium may further

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be released from metal on stainless steel implants or orthodontic appliances (Fernandez-Minano et al. 2011; Hedberg et al. 2011; Meyskens and Yang 2011; Pereira et al. 1999). Chromium pollution is a major health hazard in distinct mining areas (Das and Singh 2011). Chromium (VI) may be absorbed in the intestine and triggers oxidative stress, inflammation, cell proliferation, malignant transformation, growth arrest, cytotoxicity, and apoptosis (Chiu et al. 2010; Holmes et al. 2008; McCarroll et al. 2010; Nickens et al. 2010; Stout et al. 2009; Thompson et al. 2011; Wise et al. 2008; Yao et al. 2008). Mechanisms implicated in chromium (VI) toxicity include mitochondrial damage as well as DNA damage including base modification, single-strand breaks, double-strand breaks, Cr–DNA adducts, DNA–Cr–DNA adducts, protein–Cr–DNA adducts, and mutagenesis (Asatiani et al. 2010; Chiu et al. 2010; Hartwig 1995; Wise et al. 2008).

Erythrocytes lack mitochondria and nuclei (Lang et al. 2008), key organelles in the machinery leading to apoptosis. Nevertheless, erythrocytes can undergo eryptosis, an apoptosis-like suicidal cell death, characterized by cell membrane scrambling and cell shrinkage (Lang et al. 2008). Eryptosis is initiated by Ca^{2+} entry through Ca^{2+}-permeable cation channels (Foller et al. 2008, 2009b). The increased cytosolic Ca^{2+} activates Ca^{2+}-sensitive K^+ channels (Brugnara et al. 1993) with subsequent exit of KCl along with osmotically obliged water thus resulting in cell shrinkage (Lang et al. 2003b). Increased cytosolic Ca^{2+} activity further stimulates cell membrane scrambling with subsequent exposure of phosphatidylserine at the cell surface (Berg et al. 2001). The erythrocytes are sensitized to the cell membrane scrambling effects of Ca^{2+} by ceramide (Lang et al. 2010), which is generated by acid sphingomyelinase (Lang et al. 2005). Further triggers of eryptosis include energy depletion (Klarl et al. 2006). Beyond that, erythrocyte cell membrane scrambling may be stimulated by caspases (Bratosin et al. 2001; Mandal et al. 2002), which are activated by oxidative stress but are not necessary for Ca^{2+} induced cell membrane scrambling (Berg et al. 2001).

The present study explored, whether chromium (VI) is capable to trigger eryptosis, and if so, whether the chromium (VI) induced eryptosis involves alterations of cytosolic Ca^{2+} activity, cytosolic ATP levels and/or ceramide formation.

Materials and methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated in vitro at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h or in serum as indicated. Where indicated, erythrocytes were exposed to potassium dichromate ($K_2Cr_2O_7$; Sigma, Freiburg, Germany) and concentrations refer to those of chromium (VI) ions. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). In another experiment, $CaCl_2$ was removed and no chelating agent added to the solution. Following pretreatment with the respective Ca^{2+} containing or Ca^{2+} free Ringer solutions for the indicated time periods the erythrocytes were washed with Ringer solution containing 5 mM $CaCl_2$ and resuspended for 20 min with the same solution containing FITC-conjugated Annexin V. The time period of 20 min is considered too short for the triggering of eryptosis.

FACS analysis of annexin V binding and forward scatter

After incubation under the respective experimental condition, 50 µl cell suspensions were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with FITC-conjugated Annexin-V (1:200 dilution; Immuno-Tools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+} after incubation

After incubation 50 µl suspension erythrocytes were washed in Ringer solution and then loaded with

Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Determination of intracellular ATP concentration

For determination of intracellular erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without chromium (VI) (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

Determination of ceramide formation

For the determination of ceramide abundance, a monoclonal antibody-based assay was used. After incubation with and without chromium (VI), cells were stained for 1 h at 37°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analysed by flow cytometric analysis in FL-1.

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at $400 \times g$, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis. As Hb may leak out without erythrocytes being completely hemolysed, the method provides a maximal estimate of hemolysis.

Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 μl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μl Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally re-suspended in 50 μl of Ringer solution containing 5 mM $CaCl_2$. 20 μl were mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptotic effects, the same erythrocyte specimens have been used for control and experimental conditions.

Results

In a first series of experiments cytosolic Ca^{2+} concentration was estimated from Fluo3 fluorescence in FACS analysis. As illustrated in Fig. 1, treatment of human erythrocytes with chromium (VI) at a

concentration of ≥10 μM was followed by a statistically significant increase of Fluo3 fluorescence, an observations pointing to an increase of cytosolic Ca^{2+} concentration.

An increase of cytosolic Ca^{2+} concentration activates erythrocyte Ca^{2+}-sensitive K^+ channels leading to exit of KCl with osmotically obliged water and thus to cell shrinkage (Lang et al. 2008). A second series of experiments thus explored the effect of chromium (VI) on forward scatter in FACS analysis, a measure of cell volume. As shown in Fig. 2, at a concentration of ≥10 μM chromium (VI) treatment was indeed followed by a decrease of forward scatter.

An increased cytosolic Ca^{2+} activity further triggers cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface (Lang et al. 2008). Thus, a third series of experiments employed annexin V binding to identify phosphatidylserine exposing erythrocytes. As a first step, fluorescent annexin V was visualized by confocal imaging. As illustrated in Fig. 3, a 48 h exposure to 20 μM chromium (VI) was indeed followed by the appearance of an increased number of annexin V positive erythrocytes. In a second step annexin V binding was quantified by FACS analysis. As shown in Fig. 4a and b, a 48 h treatment with ≥10 μM chromium (VI) indeed increased the percentage of annexin V binding erythrocytes.

A further series of experiments was performed to elucidate hemolysis. To this end, hemoglobin release into the supernatant was quantified in erythrocytes exposed for 48 h to Ringer solution without or with 1–20 μM chromium (VI). As shown in Fig. 4b, treatment with ≥10 μM chromium (VI) resulted in hemolysis. The percentage of hemolytic erythrocytes was, however, one order of magnitude lower than the percentage of annexin V binding erythrocytes.

Further experiments explored whether the triggering of cell membrane scrambling by chromium (VI) required Ca^{2+} entry from the extracellular space. To this end, erythrocytes were exposed to 20 μM chromium (VI) either in the presence or in the nominal absence of extracellular Ca^{2+}. As illustrated in Fig. 4c, the effect of chromium (VI) on annexin V-binding was significantly blunted in the nominal absence of Ca^{2+}. In the nominal absence of Ca^{2+} the percentage of annexin V binding erythrocytes still tended to be slightly higher in the presence than in the absence of 20 μM chromium (VI), a difference, however, not reaching statistical significance. In a

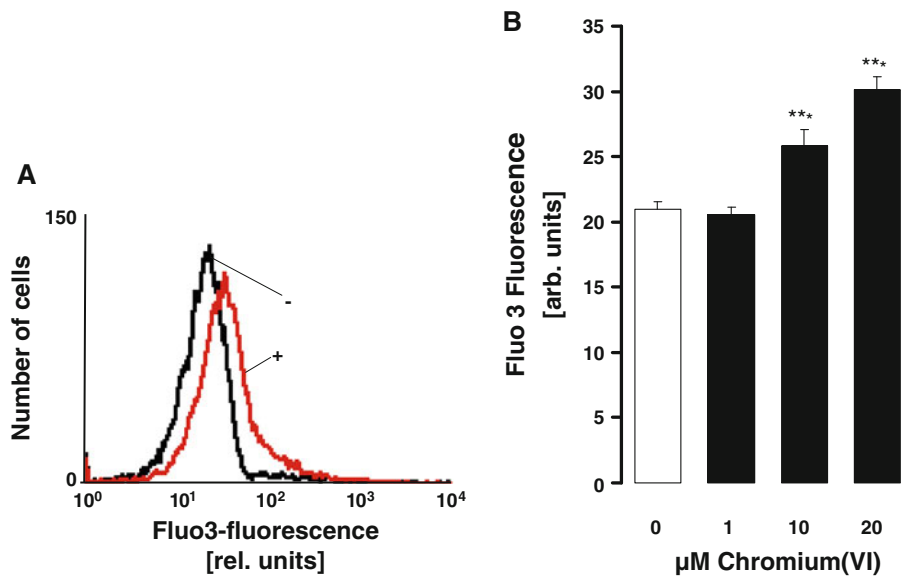

Fig. 1 Effect of chromium (VI) on Fluo3 fluorescence in erythrocytes. **a** Representative histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−) and with (+) presence of 20 μM chromium (VI). **b** Arithmetic means ± SEM ($n = 15$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (*white bar*) or with (*black bars*) 1–20 μM chromium (VI). $***P < 0.001$ indicates significant difference from the absence of chromium (VI) (ANOVA)

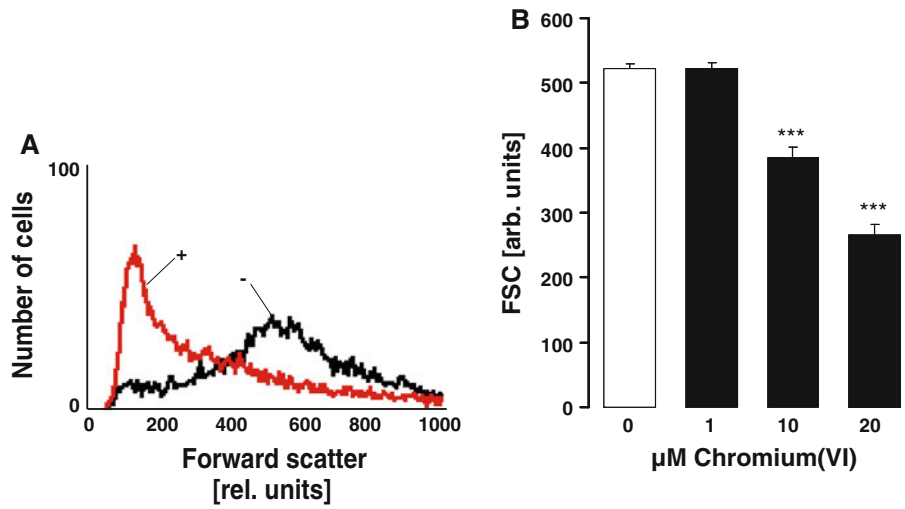

Fig. 2 Effect of chromium (VI) on erythrocyte forward scatter. **a** Representative histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (−) and with (+) presence of 20 µM chromium (VI). **b** Arithmetic means ± SEM ($n = 15$) of the erythrocyte forward scatter following incubation for 48 h to Ringer solution without (white bar) or with (black bars) 1–20 µM chromium (VI). $***P < 0.001$ indicates significant difference from the absence of chromium (VI) (ANOVA)

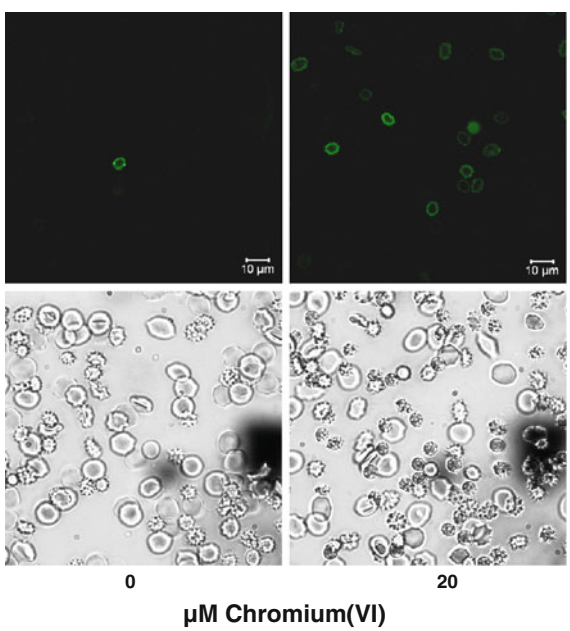

Fig. 3 Confocal images of phosphatidylserine-exposing erythrocytes with or without chromium (VI) treatment. Confocal microscopy of FITC-dependent fluorescence (*upper panels*) and light microscopy (*lower panels*) of human erythrocytes stained with FITC-conjugated annexin-V following 48 h incubation in Ringer solution without (*left panels*) and with (*right panels*) 20 µM chromium (VI)

second series of experiments, 1 mM extracellular Ca^{2+} was replaced by 1 mM EGTA (data not shown). Again, the percentage of annexin V binding erythrocytes tended to be slightly higher in the presence than in the absence of 20 µM chromium (VI), a difference again not reaching statistical significance. The effect of chromium (VI) on cell membrane scrambling is thus at least in large part secondary to an increase of intracellular Ca^{2+} activity.

Additional experiments were performed to investigate whether chromium (VI) would similarly induce phosphatidylserine exposure in erythrocytes incubated in serum. As illustrated in Fig. 4d, a 48 h treatment with 20 µM chromium (VI) increased annexin V binding to a similar extent in erythrocytes incubated in serum as in erythrocytes incubated in Ringer solution.

As eryptosis may be stimulated by energy depletion (Klarl et al. 2006), an additional series of experiments was performed to explore, whether chromium (VI) treatment influences cytosolic ATP concentration. As shown in Fig. 5, the cytosolic ATP concentration was significantly lower in erythrocytes incubated for 48 h in Ringer containing 20 µM chromium (VI) than in erythrocytes exposed for 48 h in Ringer without chromium (VI). As a positive control, cytosolic ATP concentration was determined in erythrocytes following exposure to glucose-free Ringer. As illustrated in

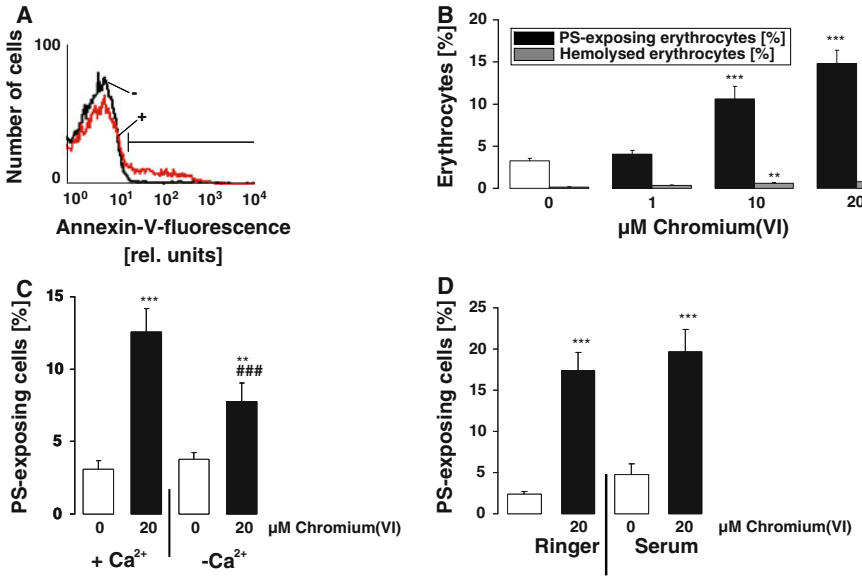

Fig. 4 Effect of chromium (VI) on phosphatidylserine exposure. **a** Representative histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without (−) and with (+) presence of 20 µM chromium (VI). **b** Arithmetic means ± SEM ($n = 15$) of erythrocyte annexin V binding following incubation for 48 h to Ringer solution without (*white bar*) or with (*black bars*) presence of 1–20 µM chromium (VI). For comparison, arithmetic means ± SEM ($n = 4$) of the percentage of hemolysis is shown as *grey bars*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$ indicates significant difference from the absence of chromium (VI) (ANOVA). **c** Arithmetic means ± SEM ($n = 8$) of the percentage of annexin V-binding erythrocytes after a 48 h treatment with Ringer solution without (*white bar*) or with (*black bars*) 20 µM chromium (VI) in the presence (*left bars*, $+Ca^{2+}$) and absence (*right bars*, $-Ca^{2+}$) of calcium. $^{**}P < 0.01$, $^{***}P < 0.001$ indicates significant difference from the absence of chromium (VI) (ANOVA), $^{\#\#\#}P < 0.001$ indicates significant difference from the respective values in the presence of Ca^{2+}. **d** Arithmetic means ± SEM ($n = 4$) of the percentage of annexin V-binding erythrocytes after a 48 h treatment with Ringer solution (*left bars*) or serum (*right bars*) without (*white bar*) or with (*black bars*) 20 µM chromium (VI). $^{***}P < 0.001$ indicates significant difference from the absence of chromium (VI) (ANOVA)

Fig. 5, glucose depletion similarly resulted in a profound decrease of ATP concentration.

A final series of experiments explored the effect of chromium (VI) treatment on the formation of ceramide, which is similarly known to trigger cell membrane scrambling (Lang et al. 2008). Ceramide formation was quantified utilizing FITC-labelled anti-ceramide antibodies. The ceramide-dependent fluorescence was, however, similar following a 48 h incubation in Ringer solution without (23.5 ± 1.9 a.u., $n = 4$) and with (21.3 ± 2.4 a.u., $n = 4$) 20 µM chromium (VI).

Discussion

The present observation unravels a novel effect of chromium (VI), i.e. the stimulation of suicidal erythrocyte death or eryptosis, characterized by stimulation of cell membrane scrambling and induction of cell shrinkage. Chromium (VI) further leads to hemolysis, an effect, however, affecting only a small percentage of erythrocytes. The concentration required for the effect on cell membrane scrambling is well in the range of concentrations observed during chromium intoxication in vivo (Thompson et al. 2011). While in healthy individuals, chromium serum concentrations are less than 0.1 µM (Chernecky and Berger 1997), values as high as 42 µM have been observed in severe chromium intoxication (Schiffl et al. 1982).

The effect of chromium (VI) on cell membrane scrambling is most likely in large part a result of Ca^{2+} entry with subsequent increase of cytosolic Ca^{2+} activity. Presumably, chromium (VI) leads to activation of the unselective cation channel, which has previously been shown to somehow involve TRPC6 (Foller et al. 2008). The cation channel is known to be activated by oxidative stress (Brand et al. 2003), a well known effect of chromium (VI) (McCarroll et al.

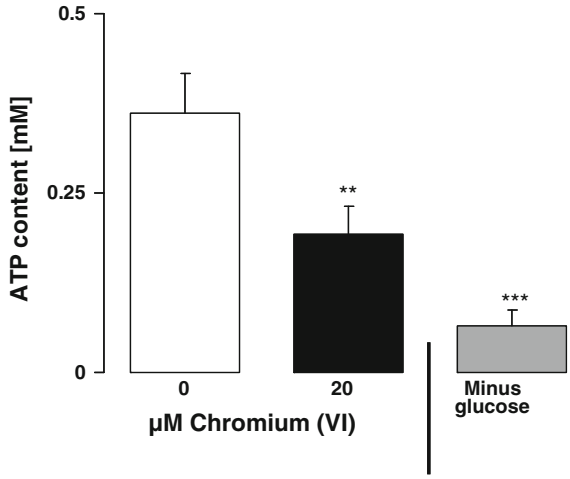

Fig. 5 Effect of chromium (VI) on erythrocyte cytosolic ATP content. Arithmetic means ± SEM ($n = 4$) of the ATP concentration after a 48 h incubation in Ringer solution without (*white bar*) or with (*black bars*) 20 μM chromium (VI), or in glucose-depleted Ringer solution (*grey bar*, minus glucose). $^{**}P < 0.01$, $^{***}P < 0.001$ indicates significant difference from control (absence of chromium (VI) and presence of glucose) (ANOVA)

2010; Thompson et al. 2011; Wise et al. 2008; Yao et al. 2008). As reported earlier (Berg et al. 2001; Bratosin et al. 2001; Lang et al. 2003a), an increased cytosolic Ca^{2+} activity is a powerful stimulator of cell membrane scrambling. In the nominal absence of extracellular Ca^{2+}, the scrambling effect of chromium (VI) lost statistical significance. The observation highlights the importance of Ca^{2+} entry but does not completely rule out further mechanisms involved in chromium (VI) induced eryptosis.

Further mechanisms known to trigger eryptosis include energy depletion (Klarl et al. 2006). As a matter of fact, chromium (VI) decreases cytosolic ATP concentration, an effect presumably contributing to the stimulation of eryptosis. The effect is again moderate and contributes to but does not fully account for the strong stimulation of cell membrane scrambling. Erythrocyte ATP generation is dependent on glycolysis and the effect of chromium (VI) on cytosolic ATP levels may reflect an interference with glycolytic flux.

Cell membrane scrambling is in addition stimulated by ceramide (Lang et al. 2010; Lang et al. 2004), which similarly triggers apoptosis of nucleated cells (Kornhuber et al. 2010). However, chromium (VI) did not significantly modify ceramide abundance. Thus, ceramide formation does presumably not contribute to the stimulation of erythrocyte cell membrane scrambling by chromium (VI).

In addition to its effect on cell membrane scrambling, an increased cytosolic Ca^{2+} activates Ca^{2+} sensitive K^+ channels (Bookchin et al. 1987; Brugnara et al. 1993) with resulting K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of water (Lang et al. 2003b). Along those lines, chromium (VI) does decrease forward scatter, an observation reflecting cell shrinkage.

Similar to what has been shown for several other triggers of eryptosis, most erythrocytes undergo eryptosis, not hemolysis following exposure to chromium. Thus, erythrocytes are removed from circulating blood without release of hemoglobin into plasma. Energy depletion, impaired Na^+/K^+ATPase or enhanced leakiness of the cell membrane are followed by cellular gain of Na^+ and Cl^- and osmotically obliged water with subsequent cell swelling (Lang et al. 1998). Cell swelling may eventually lead to rupture of the cell membrane with subsequent release of cellular hemoglobin, which is filtered in renal glomerula, precipitates in renal tubular fluid and occludes renal tubules (Burdmann et al. 1993; Reubi 1978). Timely eryptosis may prevent hemolysis, if it precedes cell swelling. Phosphatidylserine exposing erythrocytes are recognized by macrophages, which clear phosphatidylserine-exposing defective erythrocytes from circulating blood prior to hemolysis.

To the extent that the loss of erythrocytes cannot be compensated by enhanced erythrocyte formation, accelerated eryptosis may, however, decrease the number of circulating erythrocytes and thus lead to anemia (Lang et al. 2008). Moreover, adherence of phosphatidylserine-exposing erythrocytes to the vascular wall is expected to impair the microcirculation (Andrews and Low 1999; Closse et al. 1999; Gallagher et al. 2003; Pandolfi et al. 2007; Wood et al. 1996). Eryptotic erythrocytes further stimulate blood clotting (Andrews and Low 1999; Chung et al. 2007; Zwaal et al. 2005). Thus, excessive eryptosis may become pathophysiologically relevant.

Excessive eryptosis is observed in several clinical disorders (Lang et al. 2008), including diabetes (Calderon-Salinas et al. 2011; Maellaro et al. 2011; Nicolay et al. 2006), renal insufficiency (Myssina et al. 2003), Hemolytic Uremic Syndrome (Lang et al. 2006), sepsis (Kempe et al. 2007), sickle cell disease

(Lang et al. 2009). malaria (Bobbala et al. 2010; Foller et al. 2009a; Koka et al. 2009; Lang et al. 2007; Siraskar et al. 2010), Wilson's disease (Lang et al. 2007), iron deficiency (Kempe et al. 2006), phosphate depletion (Birka et al. 2004) and presumably metabolic syndrome (Zappulla 2008). Eryptosis is further triggered by a variety of substances (Bhavsar et al. 2010a, b; Braun et al. 2009; Eberhard et al. 2010; Ghashghaeinia et al. 2011; Mahmud et al. 2009a, b, c).

The present observations may shed additional light on the effects of chromium (VI) on nucleated cells. In view of the present observations, chromium (VI) may stimulate Ca^{2+} entry similarly in nucleated cells.

In conclusion, chromium (VI) stimulates Ca^{2+} entry and ATP depletion in erythrocytes, which in turn lead to cell membrane scrambling and cell shrinkage.

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