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INORGANIC COMPOUNDS

Arsenic-induced suicidal erythrocyte death

Hasan Mahmud · Michael Föller · Florian Lang

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Abstract Environmental exposure to arsenic has been associated with anemia, which could result from suicidal erythrocyte death or eryptosis, characterized by cell shrinkage and phosphatidylserine exposure at the erythrocyte surface. Eryptosis is triggered by increase in cytosolic Ca^{2+} concentration, ceramide and energy depletion. The present experiments explored, whether arsenic stimulates eryptosis. According to annexin V-binding, arsenic trioxide (7 μM) within 48 h significantly increased phosphatidylserine exposure of human erythrocytes without inducing hemolysis. According to forward scatter, arsenic trioxide (7 μM) significantly decreased cell volume. Moreover, Fluo3-fluorescence showed that arsenic (10 μM) significantly increased cytosolic Ca^{2+} concentration. According to binding of respective fluorescent antibodies, arsenic trioxide (10 μM) significantly increased ceramide formation. Arsenic (10 μM) further lowered the intracellular ATP concentration. Removal of extracellular Ca^{2+} or inhibition of the Ca^{2+}-permeable cation channels with amiloride blunted the effects of arsenic on annexin V-binding and cell shrinkage. In conclusion, arsenic triggers suicidal erythrocyte death by increasing cytosolic Ca^{2+} concentration, by stimulating the formation of ceramide and by decreasing ATP availability.

Keywords Cell volume · Annexin · Eryptosis · Calcium · Phosphatidylserine

H. Mahmud · M. Föller · F. Lang (✉)
Department of Physiology,
University of Tübingen, Gmelinstr. 5,
72076 Tübingen, Germany
e-mail: florian.lang@uni-tuebingen.de

Introduction

Arsenic exposure may lead to arsenic accumulation in erythrocytes (Kobayashi et al. 2007) and anemia (Heck et al. 2008). At least in theory, anemia could result from accelerated suicidal erythrocyte death or eryptosis (Lang et al. 2006a), which is characterized by exposure of phosphatidylserine at the erythrocyte surface (Berg et al. 2001; Bratosin et al. 2001). The phosphatidylserine exposure results from phospholipid scrambling of the cell membrane (Dekkers et al. 2002; Woon et al. 1999), which is stimulated by increased intracellular Ca^{2+} activity (Berg et al. 2001; Bratosin et al. 2001). The intracellular Ca^{2+} concentration is increased by Ca^{2+} entry through Ca^{2+}-permeable cation channels, which are activated by osmotic shock, oxidative stress and energy depletion (Lang et al. 2006a). Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels (Bookchin et al. 1987; Brugnara et al. 1993) leading to exit of KCl with osmotically obliged water and thus to cell shrinkage (Lang et al. 2006a). The effects of cytosolic Ca^{2+} on phospholipid scrambling are potentiated by ceramide, which is formed by a sphingomyelinase (Lang et al. 2006a). Phosphatidylserine-exposing erythrocytes are phagocytosed by macrophages and thus rapidly eliminated from circulating blood (Lang et al. 2006a). Thus, stimulation of eryptosis is followed by anemia. Indeed, several anemic conditions are paralleled by accelerated eryptosis, such as iron deficiency (Lang et al. 2006a), phosphate depletion (Lang et al. 2006a), hemolytic uremic syndrome (Lang et al. 2006b), sepsis (Kempe et al. 2007), malaria (Lang et al. 2006a), Wilson's disease (Lang et al. 2007), sickle cell disease (Hebbel 1991; Wood et al. 1996), thalassemia (Lang et al. 2006a), and glucose-phosphate dehydrogenase deficiency (Lang et al. 2006a). Moreover, eryptosis is triggered by several anemia-inducing drugs or

toxins, such as cordycepin (Lui et al. 2007), paclitaxel (Lang et al. 2006c), amantadine (Foller et al. 2008), chlorpromazine (Akel et al. 2006), cyclosporine (Niemoeller et al. 2006a), Bay-5884 (Shumilina et al. 2006), curcumin (Bentzen et al. 2007), valinomycin (Schneider et al. 2007), hemolysin (Lang et al. 2006a), listeriolysin (Foller et al. 2007), aluminium (Niemoeller et al. 2006b), lead (Lang et al. 2006a), mercury (Lang et al. 2006a), copper (Lang et al. 2007), methylglyoxal (Nicolay et al. 2006) and amyloid peptides (Nicolay et al. 2007).

The present study explored the effect of arsenic on eryptosis. Exposure of human erythrocytes to arsenic indeed increased cytosolic Ca^{2+}, stimulated the formation of ceramide, stimulated phosphatidylserine exposure and decreased erythrocyte cell volume.

Materials and methods

Volunteers

Erythrocytes were drawn from healthy volunteers. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical commission of the University of Tübingen.

Solutions

The in vitro experiments with arsenic were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$. Where indicated, arsenic trioxide (Sigma, Schnelldorf, Germany) was added to the NaCl Ringer resulting in final concentrations of arsenic from 2 to 10 µM or, C_6 ceramide and amiloride (both: Sigma, Schnelldorf, Germany) were added at concentrations of 50 µM and 1 mM, respectively. Caspase inhibitor zVAD was purchased from Calbiochem (Bad Soden, Germany) and applied at a concentration of 10 µM. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted for 1 mM glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA).

Measurement of hemolysis

After 48 h of incubation at 37°C in Ringer solution (composition above), the samples were centrifuged (3 min at 400 g, room temperature) 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.

Phosphatidylserine exposure and forward scatter

Erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. The cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min**,** samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analyzed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As arsenic sensitivity of different erythrocyte samples varied considerably (e.g. compare Fig. 3b, c), experiments were always designed to perform comparisons with the same blood studied at the same time with or without respective treatment.

Measurement of intracellular Ca^{2+}

Erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 20 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. To check for autofluorescence, non-stained erythrocytes were measured (Fig. 1c, left bars). Exposure of stained erythrocytes to the Ca^{2+} ionophore ionomycin (Sigma, Schnelldorf, Germany; 1 µM for 2 min) served as a positive control (Fig. 1c, right bars). Different dye loading yielded variable geo means of the fluorescence intensities of untreated and arsenic-treated erythrocytes. To avoid any bias owing to this variability, all geo means of the fluorescence intensities were normalized to the respective values of untreated erythrocytes.

Determination of ceramide formation

To determine formation of ceramide, which is exposed at the cell surface, a monoclonal antibody-based assay was used (Bieberich et al. 2003; Grassme et al. 2002) in FACS analysis. After incubation, 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. After two washing steps with PBS-BSA, 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. Samples were then analyzed by flow cytometric analysis on a FACS-Calibur in FL-1.

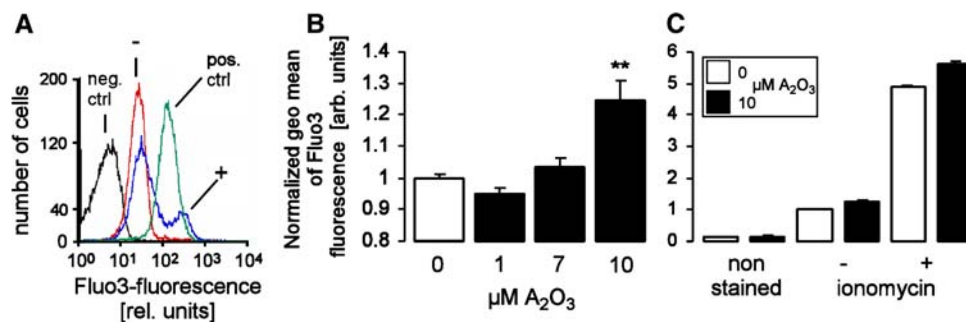

Fig. 1 Increase in cytosolic Ca^{2+} concentration in erythrocytes following exposure to arsenic. **a** Histogram of Fluo-3 fluorescence in a representative experiment of nonstained erythrocytes (negative control) or of stained erythrocytes exposed for 48 h to Ringer without (−) and with (+) 10 μM arsenic trioxide. As a positive control, stained erythrocytes were exposed to 1 μM Ca^{2+} ionophore ionomycin (positive control) for 2 min. **b** Arithmetic mean ± SEM ($n = 24$) of the normalized geo means of Fluo-3 fluorescence in erythrocytes exposed for 48 h to Ringer without (*white bar*) or with (*black bars*) arsenic. *Asterisks* indicate significant difference from the absence of arsenic (ANOVA, $P < 0.01$). **c** Arithmetic mean ± SEM ($n = 4$–24) of the normalized geo means of fluorescence in nonstained erythrocytes (*left bars*) or in Fluo-3-stained erythrocytes after a 2 min exposure to 1 μM Ca^{2+} ionophore ionomycin (*right bars*). Prior to the mentioned treatment, erythrocytes were exposed for 48 h to Ringer without (*white bars*) or with (*black bars*) 10 μM arsenic as in **b**. For comparison, the same bar diagrams as in **b** are also shown (*middle bars*)

Determination of the intracellular ATP concentration

A total of 90 μl of erythrocyte pellet was incubated for 48 h at 37°C in Ringer solution with or without arsenic (final hematocrit 5%). 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 concentration of the aliquots was 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 as mmol/l packed erythrocyte volume.

Statistics

Data are expressed as arithmetic mean ± SEM, and statistical analysis was made by paired *t* test or ANOVA, as appropriate, $P < 0.05$ was considered as statistically significant.

Results

Fluo-3 fluorescence was employed to determine, whether arsenic alters the erythrocyte Ca^{2+} concentration. As illustrated in Fig. 1a, b, Fluo-3 fluorescence significantly increased in erythrocytes following exposure to arsenic. Thus, arsenic exposure increased the intracellular Ca^{2+} concentration. The effect reached statistical significance at 10 μM arsenic trioxide concentration.

An increase in the cytosolic Ca^{2+} concentration is expected to stimulate Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl and osmotically obliged water, thus resulting in cell shrinkage (Lang et al. 2006a). Accordingly, forward scatter was determined as an estimate of cell volume. As shown in Fig. 2, exposure to arsenic indeed significantly decreased the average forward scatter, pointing to decrease of erythrocyte volume. The effect reached statistical significance at 7 μM arsenic trioxide.

An increase in the cytosolic Ca^{2+} concentration is further known to trigger scrambling of cell membrane phospholipids with subsequent exposure of phosphatidylserine at the erythrocyte surface (Berg et al. 2001; Bratosin et al. 2001). Therefore, phosphatidylserine exposure was estimated from annexin V-binding at the erythrocyte surface. As illustrated in Fig. 3a, b, treatment of erythrocytes with ≥7 μM arsenic

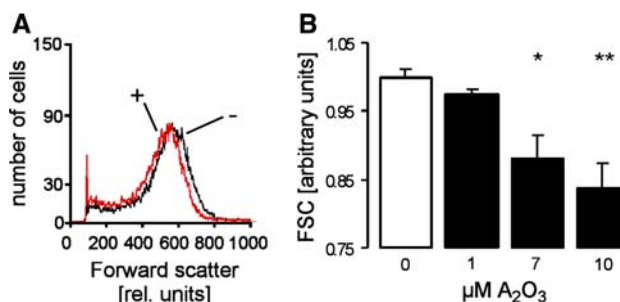

Fig. 2 Erythrocyte forward scatter following exposure to arsenic. **a** Histogram of forward scatter in a representative experiment of erythrocytes incubated for 48 h in Ringer solution (−) or in Ringer solution containing 10 μM arsenic trioxide (+). **b** Arithmetic means ± SEM ($n = 12$) of the normalized forward scatter of erythrocytes after a 48 h treatment with Ringer solution without (*white bar*) or with (*black bars*) arsenic. *Asterisks* indicate significant difference (ANOVA, $P < 0.05$, $P < 0.01$) from control (absence of arsenic)

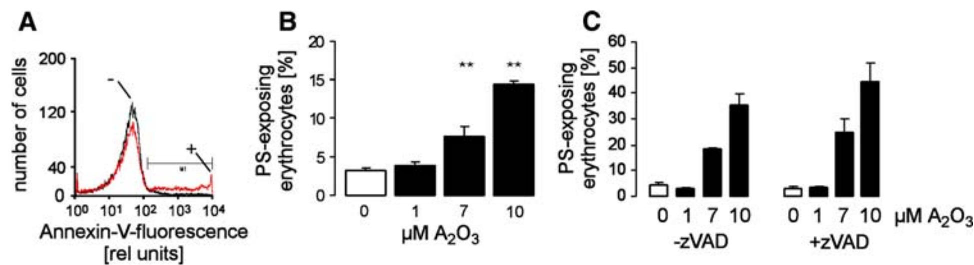

Fig. 3 Stimulation of phosphatidylserine exposure at the erythrocyte surface by arsenic. **a** Histogram of annexin V-binding in a representative experiment of erythrocytes incubated for 48 h in Ringer solution (−) or in Ringer solution containing 10 µM arsenic trioxide (+). **b** Arithmetic means ± SEM ($n = 12$) of the percentage of annexin V-binding erythrocytes after a 48-h treatment with Ringer solution without (*white bar*) or with (*black bars*) arsenic. *Asterisks* indicate significant difference (ANOVA, $P < 0.01$) from control (absence of arsenic). **c** Arithmetic mean ± 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*) arsenic in the absence (*left bars*, −zVAD) and presence (*right bars*, +zVAD) of 10 µM pancaspase inhibitor zVAD

trioxide indeed resulted in a significant increase in annexin V-binding. A 30-min treatment of erythrocytes with the Ca^{2+} ionophore (1 µM) increased the percentage of annexin-binding erythrocytes from 0.7 ± 0.2 to 26.7 ± 1.1% ($n = 8$).

Further experiments were performed to investigate a role of caspases for arsenic-induced eryptosis. However, the pancaspase inhibitor zVAD failed to inhibit arsenic-induced eryptosis (Fig. 3c).

No appreciable rate of hemolysis was observed after treatment with 10 µM arsenic trioxide (Fig. 4a). The integrity of the erythrocyte cell membrane following arsenic treatment is further illustrated by a fluorescence photograph (Fig. 4b).

Further experiments explored whether arsenic-induced cell membrane scrambling was indeed dependent on influx of Ca^{2+}. As shown in Fig. 5a, b, the effect of arsenic on annexin V-binding was significantly blunted in the nominal absence of Ca^{2+}. However, in the absence of Ca^{2+} the effect of arsenic was still statistically significant. Thus, increased cytosolic Ca^{2+} contributes to, but does not fully account for the stimulation of phospholipid scrambling of the erythrocyte cell membrane following exposure to arsenic. The effect of arsenic was further significantly blunted by inhibition of the Ca^{2+}-permeable cation channel with 1 mM amiloride (Fig. 5c).

In a further series of experiments, the effect of arsenic on erythrocyte forward scatter was determined to explore, whether arsenic-induced cell shrinkage was dependent on the presence of Ca^{2+}. As shown in Fig. 5c, arsenic-induced decrease of forward scatter was completely abrogated in the nominal absence of Ca^{2+}.

Cell membrane scrambling could further be triggered by ceramide (Lang et al. 2006a). Exposure of erythrocytes to 50 µM C_6 ceramide increased within 4 h the percentage of annexin binding erythrocytes from 0.5 ± 0.2 ($n = 8$) to 26.5 ± 4.4% ($n = 8$). Thus, additional experiments were performed to explore, whether arsenic stimulates ceramide formation. As illustrated in Fig. 6, the exposure to arsenic trioxide (10 µM) significantly increased ceramide formation. Thus, ceramide could contribute to the stimulation of cell membrane scrambling by arsenic.

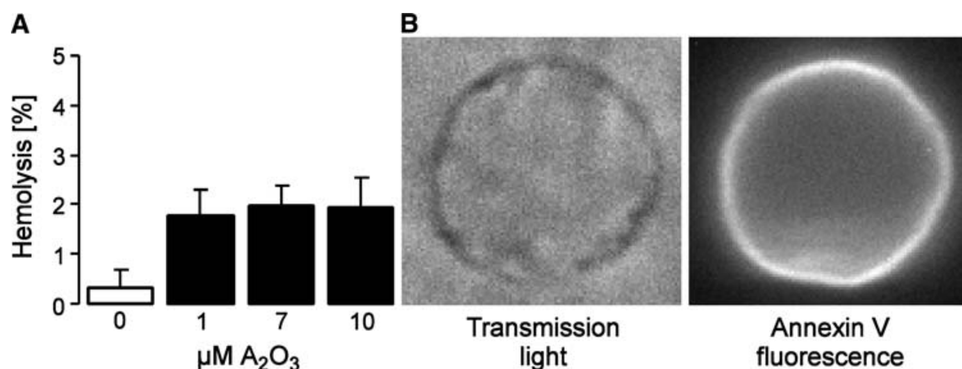

Fig. 4 Analysis of the integrity of the erythrocyte membrane under the influence of arsenic. **a** Arithmetic means ± SEM ($n = 15$) of the percentage of hemolyzed erythrocytes exposed for 48 h to Ringer solution without (*white bar*) or with (*black bars*) arsenic at the indicated concentrations. **b** Transmission microphotograph (*left panel*) and fluorescence microphotograph (*right panel*) of an erythrocyte stained with fluorescent annexin V. Prior to microscopy, the erythrocytes were exposed for 48 h to 10 µM arsenic trioxide in Ringer solution

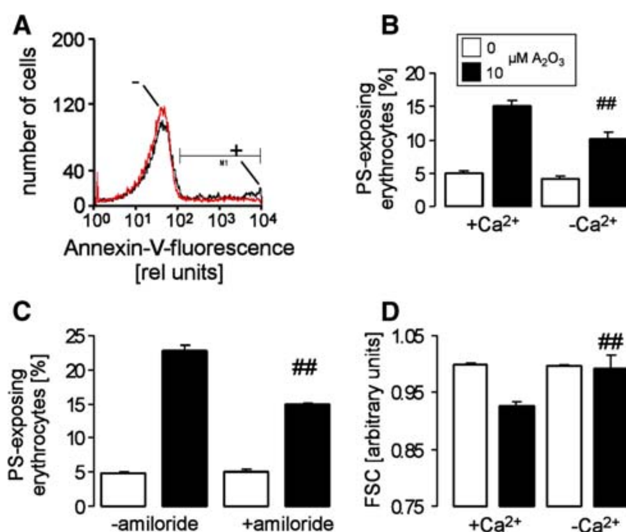

Fig. 5 Dependence of the arsenic effects on Ca^{2+} entry into erythrocytes. **a**. Histogram of annexin V-binding in a representative experiment of erythrocytes incubated for 48 h in Ringer solution containing 10 μM arsenic trioxide in the presence (+) or absence (−) of Ca^{2+}. **b** Arithmetic means ± SEM (n = 12) of the percentage of annexin V-binding erythrocytes after a 48-h treatment with Ringer solution without (*white bar*) or with (*black bars*) arsenic in the presence (*left bars*, +Ca^{2+}) and absence (*right bars*, −Ca^{2+}) of calcium. ## Significant difference (ANOVA, $P < 0.01$) from the respective values in the presence of Ca^{2+}. **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*) arsenic in the absence (*left bars*, −amiloride) and presence (*right bars*, +amiloride) of 1 mM amiloride, an inhibitor of the Ca^{2+}-permeable cation channels in erythrocytes. ## Significant difference (ANOVA, $P < 0.01$) from the respective values in the absence of amiloride. **d** Arithmetic means ± SEM (n = 12) of the normalized erythrocyte forward scatter after a 48-h treatment with Ringer solution without (*white bar*) or with (*black bars*) arsenic in the presence (*left bars*, +Ca^{2+}) and absence (*right bars*, −Ca^{2+}) of calcium. ## Significant difference (ANOVA, $P < 0.01$) from the respective values in the presence of Ca^{2+}

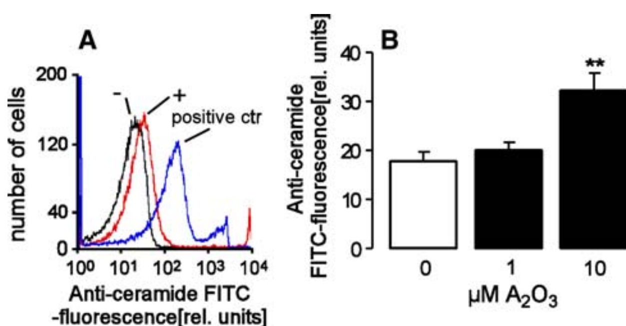

Fig. 6 Influence of arsenic on ceramide formation. **a** Histogram of ceramide abundance in a representative experiment of erythrocytes from healthy volunteers exposed for 48 h to Ringer solution without (−) or with (+) 10 μM arsenic trioxide. Exposure of erythrocytes to C_6-ceramide (50 μM) served as a positive control (positive control). **b** Arithmetic means ± SEM (n = 8) of ceramide abundance in erythrocytes exposed for 48 h to Ringer solution without (*white bar*) or with (*black bars*) arsenic at the indicated concentrations. *Asterisks* indicate significant difference from values in control Ringer solution (ANOVA, $P < 0.01$)

Since eryptosis could further be triggered by energy depletion (glucose deprivation) (Lang et al. 2006a) a further series of experiments was performed to elucidate the effect of arsenic on the erythrocyte ATP concentration. As shown in Fig. 7, the exposure to arsenic within 48 h significantly reduced the intracellular ATP concentration of erythrocytes.

Discussion

The present study discloses that arsenic leads to an increase in the intracellular Ca^{2+} concentration. Ca^{2+} then leads to the triggering of cell membrane scrambling resulting in phosphatidylserine exposure at the erythrocyte surface. Accordingly, arsenic stimulates suicidal death of erythrocytes. The effect of arsenic on phospholipid scrambling of the cell membrane is blunted by removal of extracellular Ca^{2+} and by amiloride, a known blocker of the Ca^{2+}-permeable cation channels. The effect of arsenic on phosphatidylserine exposure is, however, not completely abrogated by Ca^{2+} removal or cation channel blockade, pointing to the participation of additional mechanisms. The formation of ceramide presumably sensitizes the erythrocytes for the scrambling effect of Ca^{2+} (Lang et al. 2006a) and thus participates in the stimulation of eryptosis. In contrast, caspases are obviously not involved in the signaling of arsenic-induced eryptosis. Similarly, caspases appear not to be involved in calcium-induced erythrocyte death (Berg et al. 2001; Lang et al. 2006a). ATP depletion may contribute to the eryptotic effect of arsenic (Lang et al. 2006a). The efficacy of amiloride points to the involvement of the cation channels, which may be sensitive to ATP deficiency (Lang et al. 2006a).

In contrast, the increase in the cytosolic Ca^{2+} concentration fully accounts for the decrease of forward scatter, reflecting cell shrinkage. Ca^{2+} activates Ca^{2+}-sensitive K^+ channels (Bookchin et al. 1987; Brugnara et al. 1993), leading to exit of positively charged K^+, hyperpolarization of the cell membrane, Cl^- exit and thus cellular loss of KCl along with osmotically obliged water.

The present study did not address the mechanism activating Ca^{2+} entry and stimulating ceramide formation. It is noteworthy, though, that arsenic induces oxidative stress (Gonsebatt et al. 2007; Manna et al. 2008), which in turn is known to activate the Ca^{2+}-permeable cation channels (Lang et al. 2006a).

Phospholipid scrambling and cell shrinkage are similarly hallmarks of apoptosis of nucleated cells. Thus, the present observations may mirror similar events in nucleated cells following arsenic exposure. As a matter of fact, arsenic has been shown to inhibit cell proliferation (Conde et al. 2007) and to trigger apoptosis (Cheng et al. 2006; de la Fuente et al. 2002; Wang et al. 1998).

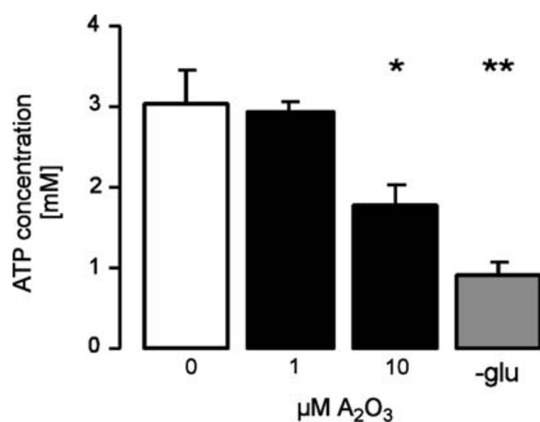

Fig. 7 Effect of arsenic on the erythrocyte ATP concentration. Arithmetic means ± SEM ($n = 4$) of the ATP concentration in erythrocytes from healthy volunteers incubated for 48 h in Ringer solution without (*white bar*) or with (*black bars*) arsenic at the indicated concentrations. As a positive control, erythrocytes were incubated in Ringer solution without glucose (−glu, *gray bar*). *Asterisks* indicate significant difference from values in control Ringer solution (ANOVA, $P < 0.05$, $P < 0.01$)

Phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood (Lang et al. 2006a) as they are bound to phosphatidylserine receptors on macrophages (Fadok et al. 2000), which engulf and degrade phosphatidylserine-exposing cells (Boas et al. 1998). Accordingly, the stimulation of phosphatidylserine exposure by arsenic accelerates erythrocyte clearance from circulating blood. Accordingly, the stimulation of suicidal death of circulating erythrocytes could cause anemia.

The arsenic concentrations needed to elicit cell membrane scrambling and cell shrinkage are close to values approached in vivo (Abdelghani et al. 1986; Shen et al. 2001; Wu et al. 2001). As a matter of fact, a most recent study demonstrated in vivo stimulation of eryptosis by contamination of drinking water (Biswas et al. 2008). Thus, eryptosis could well contribute to the anemia in arsenic intoxication. Beyond that, phosphatidylserine-exposing erythrocytes could adhere to the vascular wall (Andrews and Low 1999; Closse et al. 1999; Gallagher et al. 2003) and thus contribute to hemostasis (Andrews and Low1999). Along those lines, arsenite has been shown to enhance phosphatidylserine exposure of platelets (Bae et al. 2007). Thus, phosphatidylserine exposing cells may interfere with microcirculation.

In conclusion, exposure of erythrocytes to arsenic triggers phospholipid scrambling with phosphatidylserine exposure at the surface of the cell membrane. The effect is due to both, increased cytosolic Ca^{2+} activity and ceramide formation. The erythrocyte cell membrane scrambling is expected to accelerate clearance of erythrocytes from circulating blood leading to anemia.

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