Monensin Induced Suicidal Erythrocyte Death

Shefalee K. Bhavsar, Matthias Eberhard, Diwakar Bobbala and Florian Lang

Department of Physiology, University of Tübingen, Germany

Key Words

Phosphatidylserine • Monensin • Scrambling • Calcium • Cell volume • Eryptosis • Glucose depletion • Apoptosis

Abstract

Eryptosis, the suicidal erythrocyte death, is characterized by cell membrane scrambling and cell shrinkage. Eryptosis may be triggered by excessive hyperosmotic or isosmotic cell shrinkage leading to increase of cytosolic Ca^{2+} concentration. Eryptosis is further stimulated by the K^+ ionophore valinomycin, which leads to exit of KCl and osmotically obliged water, or by energy (glucose) depletion, which compromises the function of the Na^+/K^+ ATPase thus increasing cytosolic Na^+ concentration. The present study explored whether the Na^+ ionophore monensin affects erythrocyte cell volume and eryptosis. The cell membrane scrambling was estimated from binding of annexin V to phosphatidylserine at the erythrocyte surface, cell volume from forward scatter in FACS analysis, cytosolic Ca^{2+} concentration from Fluo3 fluorescence and the cytosolic ATP concentration from a luciferase-based assay. Within 24 hours, exposure to monensin (0.1–10 $\mu\text{g}/\text{ml}$) significantly increased forward scatter, cytosolic Ca^{2+} concentration and

annexin V-binding. Glucose depletion was followed by decreased forward scatter and increased cytosolic Ca^{2+} concentration and annexin V-binding. The effect on forward scatter was partially reversed, the effect on cytosolic Ca^{2+} concentration and annexin V binding augmented by additional treatment with monensin. In conclusion, monensin dissociates the alterations of cell membrane and cell volume in suicidal erythrocyte death.

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Introduction

Similar to apoptosis of nucleated cells the suicidal erythrocyte death or eryptosis is characterized by cell membrane scrambling and cell shrinkage [1]. Both events are triggered by increase of cytosolic Ca^{2+} concentration due to Ca^{2+} entry through Ca^{2+} -permeable cation channels [2–9]. The enhanced cytosolic Ca^{2+} concentration activates Ca^{2+} -sensitive K^+ channels [10, 11], resulting in exit of KCl with osmotically obliged water and thus in cell shrinkage [12]. Increased Ca^{2+} concentration further stimulates phospholipid scrambling of the erythro-

cyte membrane with exposure of phosphatidylserine at its surface [9, 13–16]. Erythrocytes are sensitized to the scrambling effect of Ca^{2+} by ceramide [17]. Phosphatidylserine-exposing erythrocytes are phagocytosed and rapidly cleared from circulating blood thus leading to anemia [18–20].

While excessive cell shrinkage is well known to trigger eryptosis [21], little is known about the effect of cell swelling. In nucleated cells, Na^+ entry and subsequent cell swelling may be elicited by monensin (rumensin), a well known Na^+ ionophore, which thus stimulates necrosis rather than apoptosis [22, 23]. The effect of monensin may be due to mitochondrial damage [23] and/or weakening of the antioxidative defence [24]. Monensin intoxication leads to severe rhabdomyolysis and acute renal failure with ultimate death of the patients [22, 25]. The excessive Na^+ entry following monensin intoxication stimulates the Na^+, K^+ -ATPase [26], which may in turn lead to energy depletion, another well known trigger of eryptosis [27].

The present study explored the effect of monensin on erythrocyte cell volume and cell membrane asymmetry. As a result, monensin treatment of erythrocytes leads to cell membrane scrambling and cell swelling and thus dissociates the alterations of the cell membrane and cell volume during erythrocyte death.

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 24 hours. Where indicated, monensin (Axxora, Lörrach, Germany) was added at the indicated concentrations. In Ca^{2+} -free Ringer, 1 mM CaCl_2 was substituted for 1 mM ethylene glycol tetraacetic acid (EGTA).

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, 50 μl cell suspension were washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-Fluos (1:500 dilution; Roche, Mannheim, Germany) in this solution for 20 min under protection from light. In the following, the forward scatter 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 50 μl erythrocyte suspension 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.

Measurement of hemolysis

After 24 hours of incubation at 37°C, the samples were centrifuged (3 min at 400 g, RT), and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Determination of intracellular ATP concentration

For determination of erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 24 h at 37°C in Ringer solution with or without monensin (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 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.

Statistics

Data are expressed as arithmetic means $\pm$ 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, paired comparison was employed.

Results

Treatment of erythrocytes with a Na^+ ionophore is expected to enhance the entry of Na^+ together with Cl^- and osmotically obliged water leading to cell swelling. Alterations of erythrocyte volume should be reflected by the respective changes of forward scatter (FSC) in FACS analysis. As illustrated in Fig. 1, exposure of erythrocytes

Fig. 1. Effect of monensin on erythrocyte forward scatter. A. Original histogram of the forward scatter of erythrocytes following exposure for 24 hours to Ringer solution without (-, black line) and with (+, red line) 1 µM monensin. B. Arithmetic means $\pm$ SEM ($n = 8$) of erythrocyte forward scatter following exposure for 24 hours to Ringer solution without (white bar) or with (black bars) monensin at the indicated concentrations. *, *** ($p < 0.05$, $p < 0.001$) indicates significant difference from the respective value without exposure to monensin. C. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of hemolysed erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) monensin at the indicated concentrations.

for 24 hours to Ringer solution with monensin ($= 0.1 \mu\text{M}$) was indeed followed by an increase of FSC, an effect reaching statistical significance at higher monensin concentrations (1 µM; Fig. 1). Additional experiments

Fig. 2. Effect of monensin on erythrocyte ATP content. Arithmetic means $\pm$ SEM ($n = 4$) of the ATP concentration after a 24 hours incubation in Ringer solution without (white bar) or with (black bars) monensin at the indicated concentrations or in glucose free Ringer solution (mGlu, grey bar) as a positive control. *** indicate significant difference ($p < 0.001$) from control (absence of monensin and presence of glucose).

Fig. 3. Effect of monensin on cytosolic Ca^{2+} concentration in erythrocytes. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 24 hours to Ringer solution without (-, black line) and with (+, red line) 1 µM monensin. B. Arithmetic means $\pm$ SEM ($n = 8$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 24 hours to Ringer solution without (white bar) or with (black bars) monensin. **, *** indicates significant difference ($p < 0.01$, $p < 0.001$) from the respective value in the absence of monensin.

were performed to explore, whether the increase of cell volume was sufficient to trigger hemolysis. As demonstrated in Fig. 1, at the monensin concentrations and exposure times, monensin did not elicit significant hemolysis.

Fig. 4. Effect of monensin on phosphatidylserine exposure of erythrocytes. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 24 hours to Ringer solution without (-, black line) and with (+, red line) 1 μ M monensin, M1 is marker indicating phosphatidylserine exposing cells. B. Arithmetic means $\pm$ SEM (n = 8) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 24 hours to Ringer solution without (white bar) or with (black bars) monensin. *, *** (p<0.05, p<0.001) indicates significant difference from the respective value without exposure to monensin. C. Arithmetic means $\pm$ SEM (n = 6) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 24 hours to Ringer solution in the presence (+Ca²⁺, left bars) or absence (-Ca²⁺, right bars) of extracellular Ca²⁺ without (white bars) or with (black bars) 1 μ M monensin. * (p<0.05) indicates significant difference from the respective value without exposure to monensin. ## (p<0.001) indicates significant difference from the respective value in the presence of extracellular Ca²⁺.

Excessive Na⁺ entry is expected to stimulate Na⁺/K⁺ ATPase activity, which should enhance the ATP consumption and thus decrease cytosolic ATP concentration. Accordingly, additional experiments were performed to determine, whether exposure to monensin influences ATP concentrations in erythrocytes. As shown in Fig. 2, a 24 hours exposure of human erythrocytes to monensin ($\geq 5 \mu$ M) led to a significant decrease of ATP concentration. Energy depletion by incubation in the glucose free Ringer solution (mGlu) also led to significant decrease of ATP concentration.

ATP depletion is known to increase cytosolic Ca²⁺ concentration in erythrocytes [28]. Thus, Fluo 3 fluorescence has been utilized to elucidate whether monensin influences erythrocyte Ca²⁺ concentration. As shown in Fig. 3, monensin exposure indeed increased the Fluo3 fluorescence, pointing to an increase of cytosolic Ca²⁺ concentration.

An increase in cytosolic Ca²⁺ concentration is known to stimulate cell membrane scrambling with phosphatidylserine exposure at the cell surface, which could be identified by determination of annexin V-binding. As shown in Fig. 4, the percentage of annexin V binding erythrocytes was markedly increased following exposure of erythrocytes for 24 hours to Ringer solution containing monensin (0.1 μ M to 10 μ M).

To test whether monensin-induced cell membrane scrambling is due to increase of cytosolic Ca²⁺ concentration, erythrocytes were exposed to monensin

Fig. 5. Effect of monensin on erythrocyte forward scatter following glucose depletion. A. Original histogram of the forward scatter of erythrocytes following exposure for 24 hours to glucose free Ringer solution without (-, black line) and with (+, red line) 1 μ M monensin. B. Arithmetic means $\pm$ SEM (n = 8) of erythrocyte forward scatter following exposure for 24 hours to Ringer solution (white bar) or glucose free Ringer solution (black bar) *** (p<0.001) indicates significant difference from the respective value in the presence of glucose. C. Arithmetic means $\pm$ SEM (n = 8) of erythrocyte forward scatter following exposure for 24 hours to glucose free Ringer solution without (white bar) or with (black bars) monensin at the indicated concentrations in the absence of glucose. *, ** (p<0.05, p<0.01) indicates significant difference from the respective value without exposure to monensin.

Fig. 6. Effect of monensin on cytosolic Ca^{2+} concentration in erythrocytes following glucose depletion. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 24 hours to Ringer solution without (-, black line) and with (+, red line) 1 μM monensin in the absence of glucose. B. Arithmetic means $\pm$ SEM ($n = 8$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 24 hours to Ringer without (white bar) or with (black bars) monensin in the absence of glucose. **, *** ($p < 0.01$, $p < 0.001$) indicates significant difference from the respective value in the absence of monensin. C. Arithmetic means $\pm$ SEM ($n = 8$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 24 hours to Ringer solution (white bar) or glucose free Ringer solution (black bar). ** ($p < 0.01$) indicates significant difference from the respective value in the presence of glucose. D. Arithmetic means $\pm$ SEM ($n = 8$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 24 hours to Ringer solution (white bars) or glucose free Ringer solution (black bars) in the presence of respective concentrations of monensin. **, *** ($p < 0.01$, $p < 0.001$) indicates significant difference from the respective value in the presence of glucose.

Fig. 7. Effect of monensin on phosphatidylserine exposure of erythrocytes following glucose depletion. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 24 hours to glucose free Ringer solution without (-, black line) and with (+, red line) 1 μM monensin in the absence of glucose, M1 is a marker indicating phosphatidylserine exposing cells. B. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 24 hours to Ringer solution (white bar) or glucose free Ringer solution (black bar). ** ($p < 0.01$) indicates significant difference from the respective value in the presence of glucose. C. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 24 hours to Ringer solution without (white bar) or with (black bars) monensin in the absence of glucose. *, ** ($p < 0.05$, $p < 0.01$) indicates significant difference from the respective value without exposure to monensin.

in the absence of extracellular Ca^{2+} . As shown in Fig. 4, the stimulation of annexin V binding by monensin was significantly blunted in the absence of extracellular Ca^{2+} .

ATP depletion, increase of cytosolic Ca^{2+} concentration, and subsequent stimulation of cell membrane scrambling are known features of energy depletion by omission of glucose [27]. However, glucose depletion leads to cell shrinkage rather than cell swelling [27]. Accordingly, additional experiments were performed to explore whether monensin interacts with the response of erythrocytes to glucose depletion. According to forward

scatter, exposure of the erythrocytes to glucose free solutions for 24 hours led to pronounced cell shrinkage (Fig. 5). The additional treatment with monensin partially reversed the effect of glucose depletion on forward scatter (Fig. 5).

Glucose depletion further increased cytosolic Ca^{2+} concentration in erythrocytes. As illustrated in Fig. 6, glucose depletion increased the Fluo3 fluorescence, an effect augmented in the presence of monensin. Accordingly, glucose depletion and monensin synergized to enhance cytosolic Ca^{2+} concentration.

The increase in cytosolic Ca^{2+} concentration were expected to stimulate cell membrane scrambling and thus annexin V binding. As shown in Fig. 7, the percentage of annexin V binding erythrocytes was markedly increased following glucose depletion, an effect further augmented by the presence of monensin.

Discussion

The present observations revealed that monensin swells erythrocytes, an effect expected in view of its property as Na^+ ionophore. Driven by its chemical gradient, Na^+ enters the cell, the depolarization drives Cl^- into the cell and the entry of NaCl is followed by osmotically obliged water. Monensin further decreases the cytosolic ATP concentration. The effect is presumably due to excessive ATP consumption due to stimulation of the Na^+/K^+ ATPase by increasing cytosolic Na^+ concentration. Monensin further increases cytosolic Ca^{2+} concentration, which may result from impairment of Ca^{2+} extrusion by the Ca^{2+} ATPase due to ATP deficiency. The increase of cytosolic Ca^{2+} concentration in turn contributes to or even accounts for the stimulation of cell membrane scrambling by monensin [1]. The increase of cytosolic Ca^{2+} concentration further stimulates Ca^{2+} sensitive K^+ channels thus favouring cell shrinkage [10, 11]. Thus, at lower concentrations of monensin the cell volume remains almost constant despite the Na^+ entry.

The monensin concentrations needed to trigger eryptosis do swell erythrocytes but are apparently not sufficient to trigger overt hemolysis. Thus, even though monensin leads to cell swelling, it does not trigger necrosis at the lower concentrations and shorter incubation times. At higher concentrations and extended exposure times, however, monensin is expected to trigger hemolysis and thus necrosis [22, 23].

Phosphatidylserine-exposing cells are bound to macrophages [29] and are subsequently removed by phagocytosis [30]. The rapid clearance of the phosphatidylserine exposing cells may lead to anemia [20]. Earlier studies have indeed shown that excessive eryptosis contributes to the anemia of several disorders, such as iron deficiency [20], phosphate depletion [31], Hemolytic Uremic Syndrome [32], sepsis [33], malaria [14, 34-39], or Wilson's disease [40]. Furthermore, several anemia producing xenobiotics and endogeneous substances are

at least partially effective through stimulation of eryptosis, such as cordycepin [41], amyloid peptides [42], lipopeptides [43], retinoic acid [44], amantadine [45], thymol [46], ciglitazone [47], amphotericin B [48], valinomycin [21], listeriolysin [49], copper [38], bismuth [50], tin [51], cadmium [52], selenium [53], vanadate [18], gold [54] and arsenic [55].

Besides its effect on erythrocyte survival, monensin has potent antitumor effects [56, 57]. Moreover, monensin exhibits bacteriostatic activity against a clinical isolate of *Legionella pneumophila in vitro* [58]. Monensin inhibits the mycobacterium avium subspecies paratuberculosis in radiometric culture [59]. Notably, monensin has potent *in vitro* and *in vivo* antimalarial activity [60, 61]. Eight derivatives of monensin showed potential antimalarial properties in the nanomolar range when tested *in vitro* against *Plasmodium falciparum* [62]. The antimalarial effect may be partially due to accelerated suicidal death and clearance of parasitized erythrocytes [63].

The stimulation of eryptosis may compromise microcirculation, as phosphatidylserine-exposing erythrocytes adhere to the vascular wall [64-68], and stimulate blood clotting [64, 69, 70]. Enhanced eryptosis may contribute to the vascular injury of metabolic syndrome [71]. Eryptosis might further promote the release of pro-inflammatory cytokines [71], which may in turn contribute to the described *in vivo* toxicity of monensin [22, 25].

In conclusion, monensin stimulates cell membrane scrambling of erythrocytes. In contrast to most other triggers of eryptosis it does swell cells. Thus, monensin dissociates the two hallmarks of eryptosis, i.e. cell membrane scrambling and cell shrinkage. Nevertheless, cell membrane scrambling precedes hemolysis allowing the removal of affected erythrocytes prior to disintegration of the cell membrane and release of intracellular proteins into plasma.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch and Sari Rube. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/6-1 and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research).

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