Inhibition of suicidal erythrocyte death by blebbistatin

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Submitted 11 February 2011; accepted in final form 17 May 2011

Lang E, Qadri SM, Zelenak C, Gu S, Rotte A, Draeger A, Lang F. Inhibition of suicidal erythrocyte death by blebbistatin. Am J Physiol Cell Physiol 301: C490–C498, 2011. First published May 18, 2011; doi:10.1152/ajpcell.00043.2011.—Blebbistatin, a myosin II inhibitor, interferes with myosin-actin interaction and microtubule assembly. By influencing cytoskeletal dynamics blebbistatin counteracts apoptosis of several types of nucleated cells. Even though lacking nuclei and mitochondria, erythrocytes may undergo suicidal cell death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Triggers of eryptosis include energy depletion and osmotic shock, which enhance cytosolic Ca2+ activity with subsequent Ca2+-sensitive cell shrinkage and cell membrane scrambling. The present study explored the effect of blebbistatin on eryptosis. Cell membrane scrambling was estimated from binding of annexin V to phosphatidylserine at the erythrocyte surface, cell volume from forward scatter in fluorescence-activated cell sorting analysis and cytosolic Ca2+ concentration from Fluo3 fluorescence. Exposure to blebbistatin on its own (1–50 μM) did not significantly modify cytosolic Ca2+ concentration, forward scatter, or annexin V binding. Glucose depletion (48 h) was followed by a significant increase of Fluo3 fluorescence and annexin V binding, effects significantly blunted by blebbistatin (Fluo3 fluorescence ≈ 25 μM, annexin V binding ≈ 10 μM). Osmotic shock (addition of 550 mM sucrose) again significantly increased Fluo3 fluorescence and annexin binding, effects again significantly blunted by blebbistatin (Fluo3 fluorescence ≈ 25 μM, annexin V binding ≈ 25 μM). The present observations disclose a novel effect of blebbistatin, i.e., an influence on Ca2+ entry and suicidal erythrocyte death following energy depletion and osmotic shock.

BLEBBISTATIN, a 1-phenyl-2-pyrrolidinone derivative, specifically inhibits certain isoforms of myosin II and thus interferes with myosin-actin interaction (1, 45). Blebbistatin further inhibits microtubule assembly (46). Owing to its effect on cytoskeletal dynamics blebbistatin influences the biomechanical properties of cells in a variety of tissues including skeletal muscle (1, 45), cardiac muscle (21), smooth muscle (61), endothelial cells (28, 42), renal glomerula (64), and epithelial cells (60). Blebbistatin modifies localization and function of several ion channels (29, 56) and transporters (17, 47). Blebbistatin has further been shown to prevent emulsification of mammalian erythroblasts (32) and to counteract apoptosis of several types of nucleated cells (35, 46, 48, 49, 65).

In analogy to the apoptosis of nucleated cells, erythrocytes may undergo suicidal death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage (37). Eryptosis is stimulated by an increase of cytosolic Ca2+ concentration, which may result from Ca2+-entry through Ca2+-permeable cation channels (6, 25, 30, 31, 37). Stimulators of the channels include osmotic shock and energy depletion (37, 59). Ca2+ may activate Ca2+-sensitive K+ channels (10, 13) with subsequent exit of KCl together with osmotically obliged water thus leading to cell shrinkage (37). Ca2+ further triggers cell membrane scrambling with exposure of phosphatidylserine at the cell surface (5, 11, 37). The Ca2+ sensitivity of cell membrane scrambling is enhanced by ceramide (36), which is formed by a sphingomyelinase following stimulation with platelet-activating factor (37). Erythrocyte cell membrane scrambling could be further triggered by caspases (11, 43), which are activated by oxidative stress but are not required for the scrambling effect of Ca2+ (5, 62). Similarly, CD95 appears not to be important for suicidal erythrocyte death (55).

Human red blood cells do express myosin (3) and form nonmuscle actinomyosin II complexes (26), which may well modify erythrocyte integrity, survival, and/or suicidal cell death.

The present study thus explored whether blebbistatin triggers eryptosis and/or influences the eryptosis following energy depletion or hyperosmotic shock.

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 MgSO4, 3 CaCl2, 25 glucose, and pH 7.4 at 37°C for 48 h. Where indicated, glucose was removed or extracellular osmolarity was increased by the addition of 550 mM sucrose. Where indicated blebbistatin (Sigma, Freiburg, Germany), cytochalasin B, and cytochalasin D (both Enzo, Lörrach, Germany) were added at the indicated concentrations.

Fluorescence-activated cell sorting analysis of annexin V binding and forward scatter. After incubation under the respective experimental condition, 50 μl cell suspension was washed in Ringer solution containing 5 mM CaCl2 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 (FSC) of the cells was determined, and annexin V fluorescence intensity was measured in the FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a fluorescence-activated cell sorter (FACS calibur BD, Heidelberg, Germany).

Measurement of intracellular Ca2+. After incubation, a 50-μl suspension of erythrocytes was washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution before loading.
solution containing 5 mM CaCl$_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Ca$^{2+}$-dependent fluorescence intensity was then measured in fluorescence channel FL-1 in FACS analysis.

**Determination of reactive oxygen species production.** Production of reactive oxygen species (ROS) was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 50 µl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution, and ROS-dependent fluorescence intensity was measured in the fluorescence channel FL-1 of a FACS calibur (BD).

**Estimation of the GSH/GSSG ratio.** Human erythrocytes (5% hematocrit) were incubated for 48 h at 37°C in Ringer solution and in Ringer solution without glucose in the presence and absence of 50 µM blebbistatin. The cells were washed twice in PBS. All manipulations were performed on ice. After lysis of 50 µl of the erythrocyte pellet in 250 µl distilled water and centrifugation at 14,000 rpm, 150 µl of the supernatant was deproteinated by the addition of 150 µl metaphosphoric acid (10%). Glutathione (GSSG and GSH) was measured with a glutathione assay kit (Cayman, Ann Arbor, MI) according to the manufacturer’s protocol. The GSH/GSSG ratio refers to the concentrations within erythrocytes.

**Confocal microscopy and immunofluorescence.** For the visualization of eryptotic erythrocytes, 4 µl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated Annexin-V-Fluos (1:250 dilution; Roche) in 200 µl Ringer solution containing 5 mM CaCl$_2$. Then the erythrocytes were washed twice and finally resuspended in 50 µl of Ringer solution containing 5 mM CaCl$_2$. Twenty microliters were smeared onto a glass slide and 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 ± SE. 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

As shown previously (33), energy depletion increases cytosolic Ca$^{2+}$ activity, decreases cell volume, and leads to cell membrane scrambling of erythrocytes. The present study elucidated whether those events were influenced by blebbistatin. Fluo 3 fluorescence has been used to explore whether blebbistatin influences the increase of cytosolic Ca$^{2+}$ concentration in erythrocytes following energy depletion by glucose withdrawal. As shown in Fig. 1, removal of glucose for 48 h was followed by a marked increase of cytosolic Ca$^{2+}$ concentration. Blebbistatin exposure did not significantly influence Fluo3 fluorescence in the presence of glucose. Blebbistatin
decreased, however, Fluo3 fluorescence during glucose depletion, an effect reaching statistical significance at ≥25 μM.

Ca2+ activates K+ channels with subsequent exit of KCl and cell shrinkage (10, 13, 37). Accordingly, forward scatter was determined to estimate alterations of cell volume. As shown in Fig. 2, exposure of erythrocytes for 48 h to glucose depletion was indeed followed by a sharp decrease of forward scatter. Blebbistatin did not significantly modify forward scatter in the presence of glucose.

Ca2+ further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes are identified by annexin V binding. As shown in Fig. 3, the percentage of annexin V binding erythrocytes was markedly increased following exposure of erythrocytes for 48 h to glucose-free Ringer solution. Blebbistatin did not significantly modify annexin V binding in the presence of glucose but blunted the increase of annexin V binding following glucose depletion, an effect reaching statistical significance at ≥10 μM blebbistatin (Fig. 3).

Additional experiments were performed to visualize eryptosis in the absence and presence of blebbistatin. As apparent from confocal microscopy (Fig. 4), only few erythrocytes bind annexin V in the presence of glucose. A 48-h glucose deprivation increased the number of annexin V binding erythrocytes, an effect clearly blunted in the presence of 50 μM blebbistatin (Fig. 4).

Removal of glucose from Ringer solution significantly decreased the GSH/GSSG ratio, an effect, however, not significantly modified by blebbistatin. In the presence of glucose, the GSH/GSSG ratio of erythrocytes approached 17.7 ± 5.0 (n = 4) in the absence and 11.9 ± 3.1 (n = 4) in the presence of 50 μM blebbistatin. After 48 h glucose depletion, the GSH/GSSG ratio approached 2.5 ± 0.3 (n = 4) in the absence and 3.4 ± 0.5 (n = 4) in the presence of 50 μM blebbistatin. In a further series of experiments ROS were determined utilizing DCFDA-dependent fluorescence. As a result, energy depletion significantly enhanced ROS generation and blebbistatin (50 μM) did not significantly influence ROS formation. In the presence of glucose the mean DCFDA-dependent fluorescence intensities of untreated erythrocytes was not significantly different between absence (16.2 ± 1.2 a.u., n = 4) and presence (15.2 ± 0.7 a.u., n = 4) of 50 μM blebbistatin. After 48 h glucose depletion, the mean fluorescence intensity was again similar in the absence (21.2 ± 0.5 a.u., n = 4) and presence (19.3 ± 1.4 a.u., n = 4) of 50 μM blebbistatin.

A further series of experiments explored the effect of actin depolymerization by cytochalasin B or cytochalasin D on annexin binding in the presence and absence of glucose. As a result, neither cytochalasin B nor cytochalasin D significantly modified annexin binding of glucose-repleted or glucose-depleted erythrocytes. In the presence of glucose the respective percentages of annexin binding erythrocytes were 2.5 ± 0.2%, 2.1 ± 0.1%, 2.2 ± 0.3%, and 2.1 ± 0.2% (n = 5 each), in the presence of 0, 5, 10, and 20 μM cytochalasin B, respectively, as well as 4.1 ± 0.7%, 2.7 ± 0.2%, 2.5 ± 0.1%, and 3.2 ± 1.1% (n = 5 each) in the presence of 0, 5, 10, and 20 μM cytochalasin D, respectively. After 48 h glucose depletion, the percentage of annexin binding erythrocytes approached 42.8 ±

![Figure 2](http://ajpcell.physiology.org/)

**Fig. 2.** Effect of blebbistatin on erythrocyte forward scatter following glucose depletion. A: original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without glucose, without (―, dark line) and with the (+, gray line) presence of 50 μM blebbistatin. B: arithmetic means ± SE (n = 8) of the normalized erythrocyte forward scatter following incubation for 48 h in the presence or absence of glucose in the absence (0) or presence of 1–50 μM blebbistatin. ***P < 0.001, significant difference from the presence of glucose (ANOVA).
Fig. 3. Effect of blebbistatin on phosphatidylserine exposure following glucose depletion. A: original histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without glucose, without (−, dark line) and with the (+, gray line) presence of 50 µM blebbistatin. B: arithmetic means ± SE (n = 8) of erythrocyte annexin V binding following incubation for 48 h in the presence or absence of glucose in the absence (0) or presence of 1–50 µM blebbistatin. ***P < 0.001, significant difference from the presence of glucose (ANOVA). ##P < 0.01, ###P < 0.001, significant difference from the absence of blebbistatin (ANOVA).

Fig. 4. Confocal images of phosphatidylserine-exposing erythrocytes following energy depletion in the absence and presence of blebbistatin. Confocal microscopy of FITC-dependent fluorescence (top) and light microscopy (bottom) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 h incubation in Ringer solution (left), in glucose-depleted Ringer solution (middle), and in glucose-depleted Ringer solution with 50 µM blebbistatin added (right).
6.1%, 46.0 ± 5.9%, 46 ± 7.3%, and 46.5 ± 7.3% (n = 5 each) in the presence of 0, 5, 10, and 20 μM cytochalasin B, respectively, as well as 35.8 ± 5.8%, 42.0 ± 6.1%, 40.1 ± 4.6%, and 37.8 ± 5.1% (n = 5 each) in the presence of 0, 5, 10, and 20 μM cytochalasin D, respectively.

Eryptosis is further stimulated by osmotic shock, i.e., exposure to excessive extracellular osmolarity (37). Additional experiments were thus performed to explore whether blebbistatin interferes with the effect of osmotic shock on erythrocytes.

As shown in Fig. 5, exposure of erythrocytes to hyperosmolarity (addition of 550 mM sucrose) was followed by a marked increase of cytosolic Ca²⁺ concentration. Blebbistatin exposure did not significantly influence Fluo3 fluorescence in isotonic extracellular fluid. However, blebbistatin blunted the increase of Fluo3 fluorescence following exposure of erythrocytes to osmotic shock, an effect reaching statistical significance at ≥25 μM blebbistatin.

Osmotic shock further decreased the forward scatter. As shown in Fig. 6, exposure of erythrocytes to osmotic shock was followed by a decrease of forward scatter. Blebbistatin exposure did not significantly influence forward scatter in isotonic extracellular fluid and following exposure of erythrocytes to osmotic shock (Fig. 6).

Exposure of erythrocytes to osmotic shock further increased the percentage of erythrocytes binding annexin V, pointing to an increase of phosphatidylserine exposure at the cell surface (Fig. 7). Blebbistatin did not significantly influence annexin V binding in isotonic extracellular fluid but blunted the increase of annexin V binding following exposure of erythrocytes to osmotic shock, an effect, again reaching statistical significance at ≥25 μM blebbistatin.

DISCUSSION

The present observations demonstrate that blebbistatin interferes with the known stimulating effect of energy depletion and osmotic shock on cytosolic Ca²⁺ concentration and cell membrane scrambling. Thus blebbistatin inhibits eryptosis.

The observations may shed new light on the functional role of myosin II in erythrocytes, which apparently plays an active part in triggering eryptosis. The membrane skeleton is considered to be required for keeping the membrane composition laterally homogeneous (58). Moreover, myosin II bipolar mini-filaments have been suggested to be involved in the restoration of the membrane skeleton following local mechanical or chemical damage (18). Myosin II activation may form stable links between distant antiparallel actin protofilaments leading to repair of the disrupted cell membrane (18). Moreover, myosin II may be involved in vesiculation of the bilayer (18). In some cells, apoptosis is paralleled by activation of the Rho-associated protein kinase ROCK following hyperactivation of acti-
nomyosin (48, 65). The Rho activation is coupled to Rac inhibition (48). In erythroblasts, blebbistatin-sensitive myosin is apparently involved in vesicle trafficking eventually leading to enucleation (32). Rearrangement of the cytoskeleton participates in the machinery of apoptosis (51, 54). However, the present observations do not allow the conclusion that the anti-eryptotic effect of blebbistatin is secondary to rearrangement of microfilaments or microtubules. As neither cytochalasin B nor cytochalasin D significantly modified the triggering of cell membrane scrambling following glucose depletion, actin depolymerization is not likely to play a role in the antieryptotic effect of blebbistatin.

As blebbistatin decreases the Ca$^{2+}$ entry following osmotic shock or energy depletion, myosin II may contribute to the activation of the cation channel, which mediates Ca$^{2+}$ entry. Blebbistatin has previously been shown to modify the activity of purinergic receptor channels (29, 52), connexins (53), stretch-activated channels (56), and voltage-gated Ca$^{2+}$ channels (44). The erythrocyte Ca$^{2+}$ channels have previously been shown to involve TRPC6 (24).

The effect of blebbistatin on Ca$^{2+}$ presumably accounts for its effect on eryptosis, as Ca$^{2+}$ activates Ca$^{2+}$-sensitive K$^+$ channels (10, 13) and stimulates cell membrane scrambling (5, 11, 37).

The inhibition of eryptosis by blebbistatin or related substances with more favorable pharmacological properties may be used to inhibit accelerated eryptosis. Several clinical disorders are associated with accelerated eryptosis, including iron deficiency, phosphate depletion, Hemolytic Uremic Syndrome, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, IgA-mediated human autoimmune hemolytic anemia, sepsis, sickle cell disease, malaria, Wilson’s disease, and presumably metabolic syndrome (4, 9, 14, 15, 20, 23, 34, 37, 38, 57, 66). Moreover, several xenobiotics and endogeneous substances stimulate eryptosis (7, 8, 12, 22, 39 – 41). Accelerated eryptosis leads to development of anemia (37) and phosphatidylserine-exposing erythrocytes may adhere to the vascular wall thus impeding microcirculation (2, 19, 27, 50, 63). Eryptotic erythrocytes may further stimulate blood clotting (2, 16, 67). Thus inhibition of excessive eryptosis may favorably influence the course of diseases associated with excessive eryptosis.

In conclusion, the present study reveals a novel effect of blebbistatin, i.e., inhibition of eryptosis following energy depletion and following osmotic shock.

**ACKNOWLEDGMENTS**

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch and Sari Rübe.

**GRANTS**

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).
Fig. 7. Effect of blebbistatin on phosphatidylserine exposure following osmotic shock. A: original histogram of annexin V binding of erythrocytes following a 5-h exposure to hypertonic solution (850 mosM), without (−, dark line) and with (+, gray line) the presence of 50 µM blebbistatin. B: arithmetic means ± SE (n = 8) of annexin V binding of erythrocytes following exposure for 5 h to isotonic Ringer or hypertonic (850 mosM) Ringer in the absence (0) or presence of 1–50 µM blebbistatin. 

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