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Hemin-induced suicidal erythrocyte death

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Abstract Several diseases, such as malaria, sickle cell disease, and ischemia/reperfusion may cause excessive formation of hemin, which may in turn trigger hemolysis. A variety of drugs and diseases leading to hemolysis triggers suicidal erythrocyte death or eryptosis, i.e., cell membrane scrambling and cell shrinkage. Eryptosis is elicited by increased cytosolic Ca^{2+} activity and by ceramide. The present study explored whether hemin stimulates eryptosis. Cell membrane scrambling was estimated from annexin V-binding to phosphatidylserine exposed at the cell surface, cell shrinkage from forward scatter in fluorescence-activated cell sorter analysis, cytosolic Ca^{2+} activity from Fluo3 fluorescence and ceramide formation from fluorescence-labeled antibody binding. Exposure to hemin (1–10 μM) within 48 h significantly increased annexin V-binding, decreased forward scatter, increased cytosolic Ca^{2+} activity, and stimulated ceramide formation. In conclusion, hemin stimulates suicidal cell death, which may in turn contribute to the clearance of circulating erythrocytes and thus to anemia.

Keywords Phosphatidylserine · Scrambling · Calcium · Cell volume · Eryptosis · Apoptosis

Introduction

A variety of disorders may lead to formation of hemin, which in turn may drive erythrocytes into cell death [29, 39, 48, 49, 59, 61, 70]. On the other hand, hemin has been proven useful in the treatment of acute porphyria [62, 63]. Hemin-induced damage of erythrocytes is considered to be largely due to hemolysis [48, 49, 59, 70]. However, several conditions leading to hemolysis have recently been shown to trigger eryptosis, the suicidal death of erythrocytes [40]. Hallmarks of eryptosis are scrambling of the erythrocyte membrane and cell shrinkage [40]. The cell membrane scrambling leads to exposure of phosphatidylserine at their surface, which is apparent from annexin V-binding of the erythrocytes [40]. Cell shrinkage is apparent from a decrease of forward scatter in fluorescence-activated cell sorter (FACS) analysis [40]. Eryptosis is stimulated by increase in cytosolic Ca^{2+} activity [5, 11, 12, 19, 41]. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages and are thus rapidly eliminated from circulating blood [24, 26, 36]. Cytosolic Ca^{2+} concentration is increased by activation of Ca^{2+} -permeable cation channels [20, 21, 32, 41]. The increase in cytosolic Ca^{2+} activity leads to activation of Ca^{2+} -sensitive K^+ channels [9, 14], which in turn results in exit of KCl with osmotically obliged water and thus in cell shrinkage [45]. The Ca^{2+} sensitivity of phospholipid scrambling is increased by ceramide [42].

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Fig. 1 Effect of hemin on phosphatidylserine exposure of erythrocytes. **a** Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 48 h to Ringer solution without (–, black line) and with (+, grey line) 3 μ M hemin. **b** Arithmetic means $\pm$ SEM ($n=8$) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 h to Ringer solution without (white bar) or with (black bars) hemin. *** $p<0.001$ indicates significant difference from the respective value without exposure to hemin

The present study explored whether hemin stimulates eryptosis, i.e., cell membrane scrambling and cell shrinkage of erythrocytes, and the underlying mechanism.

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, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 5 glucose, 1 CaCl₂; pH 7.4 at 37°C

for 48 h. Where indicated, hemin (Sigma, Schnellendorf, Germany) was added at the indicated concentrations.

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, cells were washed in Ringer solution containing 5 mM CaCl₂ and then stained with Annexin-V-Fluos (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 Ca²⁺

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl₂ and 2 μ M Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μ l Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Determination of ceramide formation

To determine ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1 μ g/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (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-conjugated goat anti-mouse IgG and IgM

Fig. 2 Effect of hemin on the integrity of the cellular membrane. **a** Transmission microphotograph (left panel) and fluorescence microphotograph (right panel) of an erythrocyte stained with fluorescent annexin V. Prior to microscopy, the erythrocyte was exposed for 48 h to 10 μ M hemin in Ringer solution. **b** Arithmetic means $\pm$ SEM ($n=4$)

of the percentage of hemolysed erythrocytes following exposure for 48 h to Ringer solution without (white bar) or with (black bars) hemin. *** $p<0.001$ indicates significant difference from the respective value without exposure to hemin

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 in FL-1.

Measurement of hemolysis

After 48 h of incubation at 37°C, the samples were centrifuged (3 min at 400 g, room temperature (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. To correct for absorption of hemin contained in the supernatant, the standard curve contained the same amount of hemin.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made using paired analysis of variance with Tukey's test as post-test, as appropriate. *n* denotes the number of different erythrocyte specimens studied.

Results

Cell membrane scrambling during suicidal erythrocyte death or eryptosis was detected by measurement of annexin V-binding reflecting phosphatidylserine exposure. As illustrated in Fig. 1a, b, the percentage of phosphatidylserine-exposing erythrocytes was low in the absence of hemin.

Fig. 3 Effect of hemin on erythrocyte forward scatter. **a** Original histogram of the forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (–, black line) and with (+, grey line) 3 μM hemin. **b** Arithmetic means $\pm$ SEM (*n*=10) of erythrocyte forward scatter following exposure for 48 h to Ringer solution without (white bar) or with (black bar) hemin. ****p*<0.001 indicates significant difference from the respective value without exposure to hemin

Fig. 4 Effect of hemin on cytosolic Ca²⁺ concentration in erythrocytes. **a** Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 h to Ringer without (–, black line) and with (+, grey line) 10 μM hemin **b** Arithmetic means $\pm$ SEM (*n*=10) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer without (white bar) or with (black bars) hemin. **p*<0.05, ****p*<0.001 indicate significant difference from the respective value in the absence of hemin

Exposure of erythrocytes for 48 h to Ringer solution containing hemin (1 to 10 μM) was followed by a significant increase in erythrocytic annexin V-binding. The integrity of the membrane of the majority of cells was not disrupted at 10 μM hemin (Fig. 2a). Thus, hemin only induced hemolysis of a minority of erythrocytes (Fig. 2b).

The stimulation of annexin V-binding was paralleled by a decrease of forward scatter. Exposure of erythrocytes for 48 h to Ringer solution with hemin (1 to 10 μM) was followed by a significant decrease of erythrocyte forward scatter (Fig. 3).

Further experiments explored whether hemin modifies the cytosolic Ca²⁺ concentration. To this end, the intracel-

Fig. 5 Effect of hemin on ceramide abundance at the erythrocyte membrane. **a** Histogram of ceramide-dependent fluorescence in a representative experiment of erythrocytes exposed for 48 h to Ringer without (–, black line) and with (+, grey line) 10 μM hemin. **b** Arithmetic means $\pm$ SEM (*n*=7) of the ceramide abundance following exposure for 48 h to Ringer solution without (white bar) or with (black bars) hemin. ****p*<0.001 indicates significant difference from the respective value in the absence of hemin

lular Ca^{2+} concentration of erythrocytes was determined utilizing Fluo3 fluorescence. As illustrated in Fig. 4, a 48-h incubation of erythrocytes in the presence of 1 to 10 μM hemin was followed by a significant increase in Fluo3 fluorescence, indicative of an increase in the cytosolic Ca^{2+} activity.

Another series of experiments elucidated a possible role of ceramide. As illustrated in Fig. 5, a 48-h exposure of erythrocytes to 1 to 10 μM hemin resulted in a significant increase in the ceramide abundance at the erythrocyte surface.

Discussion

The present study reveals that hemin is a potent stimulator of suicidal erythrocyte death or eryptosis. The exposure of mature human erythrocytes to hemin is followed by a significant increase in phosphatidylserine exposure and a significant decrease of forward scatter, both hallmarks of eryptosis.

A wide variety of clinical disorders is known to stimulate eryptosis, including iron deficiency [36], phosphate depletion [6], hemolytic uremic syndrome [43], sepsis [35], malaria [10, 37, 38], or Wilson's disease [46]. Some of these diseases may cause eryptosis by stimulating the formation of hemin. Furthermore, several eryptosis-triggering xenobiotics and endogenous substances have been identified, such as cordycepin [50], methylglyoxal [54], amyloid peptides [53], lipopeptides [71], retinoic acid [55], paclitaxel [44], amantadine [23], chlorpromazine [1], ciglitazone [58], cyclosporine [56], Bay-5884 [65], curcumin [4], valinomycin [64], listeriolysin [25], aluminum [57], copper [46], bismuth [13], tin [52], cadmium [68], selenium [67], vanadate [27], gold [69], and arsenic [51]. At least in theory, some of those substances may be effective through stimulation of hemin formation.

Eryptotic cells bind to phosphatidylserine receptors on macrophages [22], which engulf and degrade phosphatidylserine-exposing cells [8]. Accordingly, eryptotic cells are rapidly cleared from circulating blood [36], and stimulation of eryptosis leads to anemia, if the accelerated loss of circulating erythrocytes is not matched by a similarly increased formation of new erythrocytes. The latter is apparent from an increase in the reticulocyte number.

Eryptotic cells may further adhere to the vascular wall [3, 17, 28, 60, 72] and foster blood clotting [3, 16, 75]. Thus, phosphatidylserine-exposing erythrocytes may compromise the microcirculation. Accordingly, eryptosis has been suggested to participate in the vascular injury of metabolic syndrome [74]. The impact of hemin-induced eryptosis on microcirculation may be compounded by

effects of hemin on endothelial cells. Hemin induces oxidative stress in endothelial cells [2, 18, 33, 34]. On the one hand, hemin-induced oxidative stress may stimulate apoptosis of nucleated cells [7, 47]; on the other hand, it upregulates heme oxidase [2, 15, 18, 30], which in turn confers resistance to apoptosis [2, 30, 66]. Heme oxidase further stimulates an inflammatory response [73], which may be augmented by Hemin-induced formation of Egr-1 [31].

In conclusion, hemin stimulates suicidal erythrocyte death or eryptosis in vivo. In most patients, the effect may remain below clinical relevance. The possibility needs to be considered, however, that in particularly susceptible patients, the effect may lead to clinically relevant disturbance of microcirculation.

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