Zopolrestat Induced Suicidal Death of Human Erythrocytes

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The aldose reductase inhibitor zopolrestat has been shown to either decrease or increase apoptosis, the suicidal death of nucleated cells. Erythrocytes may similarly enter suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include oxidative stress, Ca\textsuperscript{2+} entry with increase of cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]), and ceramide formation. The present study explored, whether and how zopolrestat induces eryptosis. Methods: Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, oxidative stress from DCFDA dependent fluorescence, [Ca\textsuperscript{2+}] from Fluo3-fluorescence, and ceramide abundance utilizing specific antibodies. Results: A 48 hours exposure of human erythrocytes to zopolrestat (≥ 150 µg/ml) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter (≥ 125 µg/ml), significantly increased Fluo3-fluorescence (200 µg/ml), significantly increased ceramide abundance (150 µg/ml), but did not significantly modify DCFDA fluorescence. The effect of zopolrestat on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca\textsuperscript{2+}. Conclusions: Exposure of human erythrocytes to zopolrestat triggers cell shrinkage and cell membrane scrambling, an effect in part due to Ca\textsuperscript{2+} entry and ceramide.

Introduction

The benzothiazole zopolrestat [1], an aldose reductase inhibitor [2], considered for the treatment of diabetic neuropathy [3], has been shown to attenuate hepatic ischemia-reperfusion injury [2] and interfere with colon carcinoma cell proliferation [4]. Moreover,
zopolrestat attenuated cardiac myocyte apoptosis following hypertonic sorbitol treatment [5]. In contrast, zopolrestat enhanced the apoptosis of vascular cells, an effect attributed in part to formation of 4-hydroxynonenal, a toxic aldehyde resulting from lipid peroxidation [6].

Similar to apoptosis of nucleated cells, erythrocytes may enter 3ryptosis, a suicidal death characterized by cell shrinkage [7] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [8]. Stimulators of 3ryptosis include increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)), ceramide formation [9], energy depletion [8], and oxidative stress [8]. Signaling of 3ryptosis may further involve activated caspases [8, 10, 11] and stimulation of casein kinase 1\(\alpha\), Janus-activated kinase JAK3, protein kinase C, p38 kinase and PAK2 kinase [8]. 3ryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase and sorafenib/sunitinib sensitive kinases [8]. 3ryptosis could be triggered by a wide variety of xenobiotics [8, 12-36].

The present study explored whether zopolrestat triggers 3ryptosis. To this end, human erythrocytes from healthy volunteers were treated with zopolrestat and phosphatidylserine surface abundance, cell volume as well as [Ca\(^{2+}\)]\(_i\) and ROS formation determined by flow cytometry.

**Materials and Methods**

_Erythrocytes, solutions and chemicals_

Fresh Li-Heparin-anticoagulated blood samples 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 (184/2003 V). The blood was centrifuged at 120 x g for 20 min at 21°C and the platelets and leukocytes-containing supernatant was disposed. 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; pH 7.4), 5 glucose, 1 CaCl\(_2\) at 37°C for 48 hours. Where indicated, erythrocytes were exposed to zopolrestat (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

_Annexin-V-binding and forward scatter_

After incubation under the respective experimental condition, a 150 µl cell suspension was centrifuged at 1600 rpm for 3 mins and, after trashing the supernatant, the erythrocytes were stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in Ringer solution containing 5 mM CaCl\(_2\) at 37°C for 15 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin V binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin V-binding cells and control cells. The same threshold was used for untreated and zopolrestat treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

_Intracellular Ca\(^{2+}\)^

After incubation, a 150 µl cell suspension was centrifuged at 1600 rpm for 3 mins and, after trashing the supernatant, the erythrocytes were stained with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl\(_2\) and 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Then, Ca\(^{2+}\)-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

_Reactive oxygen species (ROS)_

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 µl suspension of erythrocytes was centrifuged at 1600 rpm for 3 mins and, after trashing the supernatant, the erythrocytes were stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min
in the dark and then washed in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, a 100 µl cell suspension was centrifuged at 1600 rpm for 3 mins, and, after trashing the supernatant, the erythrocytes were stained for 1 hour 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 1:10. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes 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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey’s test as post-test and t test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study tested, whether zopolrestat modifies eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine at the erythrocyte surface was quantified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 48 hours in Ringer solution without or with zopolrestat (50 – 200 µg/ml). As illustrated in Fig. 1, a 48 hours exposure to zopolrestat increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 150 µg/ml zopolrestat. An extended dose-response-curve is provided in Fig. 1B. Calculation of an EC50 from log [agonist] vs. normalized response (variable slope) utilizing GraphPad Prism software yielded a value of 425.4 µg/ml.

Erythrocyte volume was estimated from forward scatter, which was determined utilizing flow cytometry. As illustrated in Fig. 2, a 48 hours incubation in Ringer solution with zopolrestat (50 – 200 µg/ml) was followed by a slight decrease of forward scatter, an effect reaching statistical significance at 125 µg/ml zopolrestat concentration.

Fluo3 fluorescence was employed in order to quantify cytosolic Ca²⁺ activity ([Ca²⁺]i). As illustrated in Fig. 3, a 48 hours exposure to zopolrestat increased the Fluo3 fluorescence, an effect reaching statistical significance at 200 µg/ml zopolrestat.

In order to test whether the zopolrestat-induced translocation of phosphatidylserine required entry of extracellular Ca²⁺, erythrocytes were incubated for 48 hours in the absence or presence of 150 µg/ml zopolrestat in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 4, removal of extracellular Ca²⁺ significantly blunted the effect of zopolrestat on annexin-V-binding. Thus, zopolrestat-induced cell membrane scrambling was in large part due to entry of extracellular Ca²⁺.

Ca²⁺ entry and subsequent eryptosis is stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA). As a result, following a 48 hours incubation, the DCFDA fluorescence was not significantly different in the presence (15.8 ± 0.6 a.u., n = 5) or absence (15.3 ± 0.4 a.u., n = 5) of 150 µg/ml zopolrestat.
Fig. 1. Effect of zopolrestat on phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 200 µg/ml zopolrestat. (B) Arithmetic means ± SEM (n = 13) of erythrocyte annexin-V-binding (black bars) following incubation for 48 hours to Ringer solution without or with presence of zopolrestat (50 - 200 µg/ml). *** (P < 0.001) indicates significant difference from the absence of zopolrestat (ANOVA). (C) Percentage of annexin-V-binding erythrocytes (%) as a semilogarithmic function of zopolrestat concentration (n = 13).

Fig. 2. Effect of zopolrestat on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 200 µg/ml zopolrestat. (B) Arithmetic means ± SEM (n = 13) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without or with presence of zopolrestat (50 - 200 µg/ml). * (P<0.05), ** (P < 0.01) indicate significant difference from the absence of zopolrestat (ANOVA).
Specific antibodies were employed to quantify the ceramide abundance at the erythrocyte surface. As illustrated in Fig. 5, a 48 hours exposure to 150 µg/ml zopolrestat significantly increased the ceramide abundance.
Discussion

The present study reveals a novel effect of zopolrestat, i.e. the triggering of suicidal erythrocyte death or eryptosis. A 48 hours exposure of human erythrocytes to zopolrestat results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for the toxic effect may be approached in vivo at dosages of 800 mg/day or of 25 g/kg BW. The susceptibility to eryptosis may be increased and thus lower zopolrestat concentrations be effective in several clinical conditions, such as dehydration, hyperphosphatemia, chronic kidney disease (CKD), hemolytic-uremic syndrome, diabetes, hepatic failure, malignancy, sepsis, sickle-cell disease, beta-thalassemia, Hb-C and G6PD-deficiency, as well as Wilsons disease.

The effect of zopolrestat on cell membrane scrambling was paralleled by increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)) and was significantly blunted by removal of extracellular Ca\(^{2+}\). Thus, it appears safe concluding that the triggering of eryptosis by zopolrestat was in part due to Ca\(^{2+}\) entry with subsequent increase of [Ca\(^{2+}\)]. Ca\(^{2+}\) stimulates cell membrane scrambling by activating a scramblase, which awaits molecular identification. An increase of [Ca\(^{2+}\)] further activates Ca\(^{2+}\) sensitive K\(^+\) channels with subsequent cell shrinkage due to K\(^+\) exit, cell membrane hyperpolarization, Cl\(^-\) exit and thus cellular loss of KCl with water.

Even in the absence of extracellular Ca\(^{2+}\), however, zopolrestat leads to increase of annexin-V-binding, an observation pointing to additional mechanisms. As revealed by specific antibodies, zopolrestat treatment did increase the abundance of ceramide, which is known to trigger eryptosis at constant [Ca\(^{2+}\)]. According to DCFDA fluorescence, zopolrestat treatment did not appreciably trigger oxidative stress. The present study does not rule out the involvement of further mechanisms, such as caspase activation and or altered activity of eryptosis stimulating or eryptosis inhibiting kinases.

Eryptotic erythrocytes are rapidly cleared from circulating blood. Eryptosis may thus prevent intravascular hemolysis with release of hemoglobin, which is otherwise filtered in renal glomeruli, precipitates in the acidic lumen of renal tubules and thus occludes nephrons. Eryptosis may further counteract increase of parasitemia following infection with the malaria pathogen *Plasmodium*. The pathogen imposes oxidative stress on the infected host erythrocyte leading to opening of Ca\(^{2+}\)-permeable erythrocyte cation channels. The enhanced susceptibility of sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency...
to eryptosis accelerates the eryptosis and subsequent demise of infected erythrocytes. Accordingly, those genetic disorders decrease parasitemia and protect against a severe course of malaria [8, 49-51]. Along those lines, iron deficiency [52] and treatment with lead [52], chlorpromazine [53] or NO synthase inhibitors [53] enhance the susceptibility of erythrocytes to pathogen-induced eryptosis and thus counteract parasitemia. It is tempting to speculate that zopolrestat may similarly foster removal of infected erythrocytes and thus favourably influence the clinical course of malaria.

On the other hand, eryptosis may lead to anemia [8] and impair microcirculation [9, 54-58] by adherence of phosphatidylserine exposing erythrocytes to the vascular wall [59] as well as stimulation of blood clotting and thrombosis [54, 60, 61].

In conclusion, exposure of human erythrocytes to zopolrestat is followed by eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to Ca\(^{2+}\) entry and increased abundance of ceramide.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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