Original Paper

Simvastatin, a Novel Stimulator of Eryptosis, the Suicidal Erythrocyte Death

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Phosphatidylserine • Cell membrane scrambling • Oxidative stress • Calcium

Abstract

Background/Aims: The 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase inhibitor simvastatin has been shown to trigger apoptosis of several cell types. The substance has thus been proposed as an additional treatment of malignancy. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis include cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the extracellular face of the erythrocyte cell membrane. Signaling contributing to stimulation of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), induction of oxidative stress, increase of ceramide abundance, and activation of SB203580-sensitive p38 kinase. The present study explored, whether simvastatin induces eryptosis and aimed to shed light on cellular mechanisms involved. **Methods:** Flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from DCFDA dependent fluorescence, and ceramide abundance utilizing specific antibodies. Hemolysis was estimated from hemoglobin concentration in the supernatant. **Results:** A 48 h exposure of human erythrocytes to simvastatin (1 $\mu\text{g}/\text{ml}$) significantly decreased the forward scatter, significantly augmented the percentage of annexin-V-binding cells, significantly increased Fluo3-fluorescence, and significantly enhanced DCFDA fluorescence. Simvastatin tended to increase ceramide abundance, an effect, however, escaping statistical significance. The effect of simvastatin on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} and by addition of SB203580 (2 μM). **Conclusions:** Simvastatin stimulates eryptosis, an effect at least in part due to Ca^{2+} entry, oxidative stress, and p38 kinase.

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Published by S. Karger AG, Basel**Introduction**

Simvastatin, a 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase inhibitor decreasing cholesterol synthesis [1], is widely used for the treatment of dyslipidemia with hypercholesterolemia [2-15]. Further putative therapeutic effects include decrease of

hippocampal amyloid-beta abundance in Alzheimer's disease [16], anticoagulation [10], inhibition of inflammatory disease, such as asthma [1, 17-19], protection against contrast-induced nephropathy [20], protection of cardiac allografts against ischemia/reperfusion injury [21], as well as counteraction of osteoporosis and support of bone fracture healing [22, 23]. Side effects of simvastatin include rhabdomyolysis [6, 8, 24-29] and thrombocytopenia [30].

Simvastatin has been shown to stimulate apoptosis of neurons [31] and tumor cells [32-39]. In view of its effect in tumor cells simvastatin was considered as a potential therapeutic option in malignancy [32, 35-37]. On the other hand, simvastatin may inhibit apoptosis of osteoblasts [22, 40].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis [41], the suicidal death of erythrocytes characterized by cell shrinkage [42] and by cell membrane scrambling with phosphatidylserine translocation to the outer face of the erythrocyte [41, 43] and release of exosomes [44]. Signaling stimulating eryptosis includes increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [41, 45], ceramide [46], caspases [41, 47, 48], G-protein Galphai2 [49], casein kinase 1 α [41, 50], Janus-activated kinase JAK3 [41, 51], protein kinase C [41, 52], and p38 kinase [41, 53]. Eryptosis is suppressed by AMP activated kinase AMPK [41, 54], cGMP-dependent protein kinase [41, 55], mitogen and stress activated kinase MSK1/2 [56], PAK2 kinase [41, 57] and sorafenib/sunitinib sensitive kinases [41, 58, 59]. Eryptosis could be triggered by a wide variety of cell stressors including hyperosmotic shock [41], oxidative stress [41], energy depletion [41], and a myriad of xenobiotics [41, 60-117]. Enhanced eryptosis is observed in multiple clinical conditions including iron deficiency [41], dehydration [118], hyperphosphatemia [119], vitamin D excess [70], chronic kidney disease (CKD) [120-124], hemolytic-uremic syndrome [125], diabetes mellitus [126], hepatic failure [94, 127], malignancy [128, 129], arteritis [130], sepsis [131], sickle-cell disease [41], beta-thalassemia [41], Hb-C and G6PD-deficiency [41], and Wilsons disease [132]. Eryptosis is further enhanced in elderly individuals [133], sensitive to erythrocyte age [107], and increased following extended storage of erythrocytes for transfusion [134].

To possibly uncover an effect of simvastatin on eryptosis, human erythrocytes from healthy volunteers were exposed to simvastatin and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, reactive oxygen species (ROS) and ceramide abundance 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 g for 20 min at 21 °C and the supernatant with platelets as well as leukocytes was disposed. Erythrocytes were incubated *in vitro* at a packed cell volume 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 h. Where indicated, erythrocytes were exposed for 48 h to simvastatin (Sigma Aldrich, Hamburg, Germany). In order to estimate the impact of Ca^{2+} entry on simvastatin induced eryptosis, erythrocytes were exposed to simvastatin in the absence of extracellular Ca^{2+} . To test for an involvement of p38 kinase, erythrocytes were exposed for 48 hours to a combination of simvastatin and p38 kinase inhibitor SB203580 (Tocris bioscience, Bristol, UK).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μl cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution 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 simvastatin treated erythrocytes. The threshold of forward scatter was set at the default value of “52”.

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. 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. Afterwards, the geomean of the Ca^{2+} dependent fluorescence was determined.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μ l 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). Subsequently, the geomean of the DCFDA dependent fluorescence was determined.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, cells were stained for 1 h at 37°C with 1 μ g/ml anti ceramide antibody (clone MID 15B4, Enzo Life Science GmbH, Lörrach, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. The 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. The samples were analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

Hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 1600 rpm in room temperature) after incubation, 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.

Statistics

Data are expressed as arithmetic means $\pm$ 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

In order to test, whether simvastatin modifies eryptosis, erythrocytes drawn from healthy individuals were exposed for 48 h to Ringer solution without or with simvastatin (0.5 – 2 μ g/ml). Eryptotic erythrocytes were identified from cell shrinkage and cell membrane scrambling.

Cell volume was estimated from forward scatter in flow cytometry. As illustrated in Fig. 1A,B, a 48 h exposure to simvastatin decreased the forward scatter, an effect reaching statistical significance at 1 μ g/ml simvastatin concentration. Thus, simvastatin triggered cell shrinkage. Along those lines, at all simvastatin concentrations tested, simvastatin

Fig. 1. Effect of Simvastatin on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 2 $\mu\text{g}/\text{ml}$ Simvastatin. B. Arithmetic means $\pm$ SEM ($n = 14$) of the erythrocyte forward scatter (FSC) following incubation for 48 h in Ringer solution without (white bar) or with (black bars) Simvastatin (0.5 - 2 $\mu\text{g}/\text{ml}$) or solvent DMSO alone (grey bar). C,D. Arithmetic means $\pm$ SEM of the percentage of erythrocytes with (C) FSC < 200 or (D) FSC > 800 following incubation for 48 h to Ringer solution without (white bar) or with (black bars) Simvastatin (0.5 - 2 $\mu\text{g}/\text{ml}$) or solvent DMSO alone (grey bar). *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

Fig. 2. Effect of simvastatin on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 2 $\mu\text{g}/\text{ml}$ simvastatin. B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte annexin-V-binding following incubation for 48 h in Ringer solution without (white bar) or with (black bars) simvastatin (0.5 - 2 $\mu\text{g}/\text{ml}$). For comparison, the effect of the solvent DMSO is shown (grey bar). ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

significantly increased the percentage of shrunken erythrocytes (Fig. 1C) and significantly decreased the percentage of erythrocytes with large volumes (Fig. 1D).

Cell membrane scrambling with phosphatidylserine translocation to the cell surface was evidenced from annexin-V-binding to phosphatidylserine, as determined by flow cytometry. As illustrated by Fig. 2A,B, a 48 h exposure to simvastatin increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 1 $\mu\text{g}/\text{ml}$ simvastatin.

Eryptosis could be triggered by increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was thus employed in order to quantify $[\text{Ca}^{2+}]_i$. As illustrated in Fig. 3A,B, a 48 h exposure to simvastatin increased the Fluo3 fluorescence, an effect reaching statistical significance at 2 $\mu\text{g}/\text{ml}$ simvastatin.

In order to test, whether the increase of $[\text{Ca}^{2+}]_i$ required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 h in the absence or presence of 2 $\mu\text{g}/\text{ml}$ simvastatin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, removal of extracellular Ca^{2+} tended to blunt the effect of simvastatin on $[\text{Ca}^{2+}]_i$, an effect, however, not reaching statistical significance. Exposure of erythrocytes for 48 h to simvastatin (2 $\mu\text{g}/\text{ml}$) significantly increased the Fluo3 fluorescence both, in the presence and nominal absence of extracellular Ca^{2+} .

Fig. 3. Effect of simvastatin on cytosolic Ca^{2+} activity. A. Original histogram of Fluo3 fluorescense reflecting erythrocyte cytosolic Ca^{2+} activity following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 2 $\mu\text{g/ml}$ simvastatin. B. Arithmetic means $\pm$ SEM ($n = 14$) of erythrocyte Fluo3 fluorescence following incubation for 48 h in Ringer solution without (white bar) or with (black bars) simvastatin (0.5 - 2 $\mu\text{g/ml}$). For comparison, the effect of the solvent DMSO is shown (grey bar). C. Arithmetic means $\pm$ SEM of the percentage of erythrocytes with enhanced Fluo3 fluorescence following incubation for 48 h in Ringer solution without (white bar) or with (black bars) Simvastatin (0.5 - 2 $\mu\text{g/ml}$) or solvent DMSO alone (grey bar). *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

Fig. 4. Sensitivity of simvastatin-induced increase of cytosolic Ca^{2+} activity to presence or nominal absence of extracellular Ca^{2+} . Arithmetic means $\pm$ SEM ($n = 19$) of erythrocyte Fluo3 fluorescence following incubation for 48 hours in Ringer solution without (white bars) or with (black bars) simvastatin (2 $\mu\text{g/ml}$) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

Fig. 5. Simvastatin-induced erythrocyte shrinkage in presence or nominal absence of extracellular Ca^{2+} . Arithmetic means $\pm$ SEM ($n = 24$) of erythrocyte Fluo3 fluorescence following incubation for 48 hours in Ringer solution without (white bars) or with (black bars) simvastatin (2 $\mu\text{g/ml}$) in the presence (left bars, + Ca^{2+}) and nominal absence (right bars, - Ca^{2+}) of Ca^{2+} . **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

As illustrated in Fig. 5, a 48 h treatment of erythrocytes with 2 $\mu\text{g/ml}$ of simvastatin decreased the forward scatter significantly in both, the presence and absence of extracellular Ca^{2+} . The effect tended to be smaller in the nominal absence than in the presence of extracellular Ca^{2+} , a difference, however, not reaching statistical significance.

In order to test whether simvastatin-induced cell membrane scrambling required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 h in the absence or presence of 2 $\mu\text{g/ml}$ simvastatin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 6A-C, removal of extracellular Ca^{2+} significantly blunted the effect of simvastatin on annexin-V-binding. However, even in the nominal absence of extracellular Ca^{2+} , simvastatin significantly increased the percentage of annexin-V-binding erythrocytes. Thus, the simvastatin-induced cell membrane scrambling was in part but not completely due to entry of extracellular Ca^{2+} .

Fig. 6. Ca^{2+} sensitivity of simvastatin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey areas) and with (black lines) simvastatin (2 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means $\pm$ SEM ($n = 18$) of annexin-V-binding of erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) simvastatin (2 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of simvastatin, #($p < 0.05$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 7. Effect of simvastatin on reactive oxygen species. A. Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey area) and with (black line) presence of 2 $\mu\text{g}/\text{ml}$ simvastatin. B. Arithmetic means $\pm$ SEM ($n = 22$) of DCFDA fluorescence in erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) simvastatin (1 $\mu\text{g}/\text{ml}$). C. Arithmetic means $\pm$ SEM of the percentage of erythrocytes with enhanced DCFDA fluorescence following incubation for 48 h to Ringer solution without (white bar) or with (black bars) Simvastatin (0.5 - 2 $\mu\text{g}/\text{ml}$) or solvent DMSO alone (grey bar). ***($p < 0.01$) indicates significant difference from the absence of simvastatin (unpaired t test).

Fig. 8. Effect of simvastatin on reactive oxygen species in presence or nominal absence of extracellular Ca^{2+} . Arithmetic means $\pm$ SEM ($n = 19$) of erythrocyte DCFDA fluorescence following incubation for 48 hours in Ringer solution without (white bars) or with (black bars) simvastatin (2 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin, ##($p < 0.01$) indicates significant difference from the absence of Ca^{2+} (ANOVA).

Eryptosis is further stimulated by oxidative stress. The abundance of reactive oxygen species (ROS) was thus quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 7A,B, a 48 h exposure to simvastatin (2 $\mu\text{g}/\text{ml}$) increased the average DCFDA fluorescence of erythrocytes from 15.6 ± 0.4 a.u. ($n = 22$) to 20.8 ± 1.3 a.u.

Fig. 9. SB203580 sensitivity of simvastatin-induced phosphatidylserine exposure. A,B. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) simvastatin (2 µg/ml) in the absence (A) and presence (B) of p38 kinase inhibitor SB203580 (2 µM). C. Arithmetic means $\pm$ SEM (n = 8) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) simvastatin (2 µg/ml) in the absence (left bars, - SB203580) and presence (right bars, + SB203580) of p38 kinase inhibitor SB203580 (2 µM). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin, ###($p < 0.001$) indicates significant difference from the absence of SB203580 (2 µM) (ANOVA).

Fig. 10. Effect of simvastatin on hemolysis. Arithmetic means $\pm$ SEM (n = 5) of erythrocyte hemolysis following incubation for 48 h to Ringer solution without (white bar) or with (black bars) simvastatin (0.5 - 2 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). **($p < 0.01$), ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

Fig. 11. Simvastatin-induced hemolysis in presence or nominal absence of extracellular Ca^{2+} . Arithmetic means $\pm$ SEM (n = 10) of the percentage hemolytic erythrocytes following incubation for 48 hours in Ringer solution without (white bars) or with (black bars) simvastatin (2 µg/ml) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of simvastatin (ANOVA).

(n = 22). Moreover, simvastatin significantly increased the percentage of erythrocytes with high DCFDA fluorescence (Fig. 7C). The observations suggest that simvastatin did induce oxidative stress.

Again, oxidative stress was measured following incubation for 48 h in the absence or presence of 2 µg/ml simvastatin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 8, DCFDA fluorescence of erythrocytes significantly increased both, in the presence and absence of extracellular calcium. The average DCFDA fluorescence reached significantly higher values in the absence than in the presence of extracellular Ca^{2+} .

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As a result, the ceramide abundance tended to be higher following exposure to 2 µg/ml simvastatin (8.72 ± 0.34 a.u., n = 15) than following incubation in the absence of simvastatin (7.77 ± 0.32 a.u., n = 15), a difference, however, not reaching statistical significance.

To explore, whether the effects of simvastatin involved p38 kinase activity, the influence of simvastatin on annexin-V-binding was tested in the absence and presence of p38 kinase inhibitor SB203580. As illustrated in Fig. 9, SB203580 (2 μ M) significantly blunted the effect of simvastatin (2 μ g/ml) on annexin-V-binding. However, even in the presence of SB203580, simvastatin significantly increased the percentage of annexin-V-binding erythrocytes.

A final series of experiments explored the influence of simvastatin on hemolysis. The percentage of hemolytic cells was quantified from the hemoglobin concentration in the supernatant. As illustrated in Fig. 10, a 48 h exposure to simvastatin increased the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 1 μ g/ml simvastatin.

Again the role of extracellular Ca^{2+} was tested by incubation for 48 h in the absence or presence of 2 μ g/ml simvastatin in the presence or nominal absence of extracellular Ca^{2+} . As illustrated Fig. 11, the percentage of hemolytic erythrocytes increased significantly to virtually the same extent in the presence and absence of extracellular Ca^{2+} .

Discussion

The present observations reveal that simvastatin triggers erythrocyte shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Accordingly, simvastatin does not only trigger apoptosis, the suicidal death of nucleated cells [31-39], but by the same token triggers eryptosis, the suicidal erythrocyte death. The simvastatin concentrations required for the stimulation of eryptosis were in the range of concentrations (0.9 μ g/ml) measured in the plasma of patients [39].

The effect of simvastatin on cell volume and cell membrane scrambling was paralleled by an increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Moreover, the effect of simvastatin on cell membrane scrambling was partially blunted by removal of extracellular Ca^{2+} , an observation pointing to a role of Ca^{2+} entry in the triggering of eryptosis. However, simvastatin triggered cell membrane scrambling even in the nominal absence of extracellular Ca^{2+} , an observation suggesting the participation of additional mechanisms in simvastatin induced cell membrane scrambling. It must be kept in mind that removal of Ca^{2+} from and addition of EDTA to the Ringer solution (nominal absence of Ca^{2+}) does not completely eliminate extracellular Ca^{2+} . We cannot rule out that residual extracellular Ca^{2+} enters the cell and elicits the observed effects.

Cells could be sensitized for the scrambling effect of Ca^{2+} by ceramide [41]. Simvastatin treatment tended to increase the ceramide abundance. The effect did, however, not reach statistical significance.

Simvastatin significantly enhanced the abundance of reactive oxygen species, a well known trigger of eryptosis [41]. Simvastatin is therefore partially effective by inducing oxidative stress. Similar to the triggering effect of simvastatin on apoptosis of osteosarcoma cells [135], the stimulation of eryptosis by simvastatin on cell membrane scrambling requires apparently activity of p38 kinase, which is known to participate in the orchestration of eryptosis [53]. Accordingly, the effect of simvastatin on annexin-V-binding is significantly blunted in the presence of p38 kinase inhibitor SB203580.

The simvastatin-induced erythrocyte shrinkage was not significantly blunted in the nominal absence of extracellular Ca^{2+} . This observation does not rule out the involvement of Ca^{2+} entry. An increase of $[\text{Ca}^{2+}]_i$ activates Ca^{2+} -sensitive K^+ channels followed by K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [42]. Whether additional factors contribute to simvastatin induced erythrocyte shrinkage, remained elusive. The present experiments cannot rule out the participation of further mechanisms in the stimulation of cell membrane scrambling and cell shrinkage following simvastatin treatment of erythrocytes.

Simvastatin further triggered hemolysis, pointing to disruption of the cell membrane. The function of eryptosis is the removal of defective erythrocytes from circulating blood prior to hemolysis [41]. Without timely removal of defective erythrocytes hemoglobin released following hemolysis passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and may thus lead to renal failure [136].

The clearance of phosphatidylserine exposing erythrocytes may result in the development of anemia. A combined therapy with simvastatin and cetuximab frequently leads to anemia [137]. However, we did not find reports of anemia following treatment with simvastatin alone. Anemia develops only, when the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [41]. It should further be kept in mind that simvastatin decreases plasma concentrations of C-reactive protein (CRP) [138], a stimulator of eryptosis [139]. Thus the direct stimulation of eryptosis may be attenuated by the indirect decrease of CRP-induced eryptosis.

Besides its potential effect on anemia, eryptosis may interfere with microcirculation [46, 140-144], as phosphatidylserine exposing erythrocytes adhere to other phosphatidylserine exposing erythrocytes [145] and the vascular wall [146], stimulate blood clotting and trigger thrombosis [140, 147, 148]. The present observations may thus not only be relevant for development of anemia but may suggest to carefully consider the risk of thrombosis following simvastatin treatment. This may be particularly important in patients suffering from clinical disorders associated with enhanced eryptosis. However, the present data cannot be taken as evidence that simvastatin treatment triggers thrombosis.

In conclusion, simvastatin stimulates Ca^{2+} entry into erythrocytes, triggers oxidative stress and thus triggers erythrocyte shrinkage and erythrocyte cell membrane scrambling, the hallmarks of eryptosis, the suicidal death of erythrocytes.

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Disclosure Statement

None.

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