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Original Paper

Age Sensitivity of NFκB Abundance and Programmed Cell Death in Erythrocytes Induced by NFκB Inhibitors

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Key Words
Phosphatidylserine • Bay 11-7082 • Parthenolide • Cell volume • Eryptosis

Abstract
Background/Aims: Erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and phosphatidylserine exposure at the erythrocyte outer membrane. Susceptibility to eryptosis is enhanced in aged erythrocytes and stimulated by NFκB-inhibitors Bay 11-7082 and parthenolide. Here we explored whether expression of NFκB and susceptibility to inhibitor-induced eryptosis is sensitive to erythrocyte age. ***Methods:*** Human erythrocytes were separated into five fractions, based on age-associated characteristics cell density and volume. NFκB compared to ß-actin protein abundance was estimated by Western blotting and cell volume from forward scatter. Phosphatidylserine exposure was identified using annexin-V binding. ***Results:*** NFκB was most abundant in young erythrocytes but virtually absent in aged erythrocytes. A 24h or 48h exposure to Ringer resulted in spontaneous decrease of forward scatter and increase of annexin V binding, effects more pronounced in aged than in young erythrocytes. Both, Bay 11-7082 (20 μM) and parthenolide (100 μM) triggered eryptosis, effects again most pronounced in aged erythrocytes. ***Conclusion:*** NFκB protein abundance is lowest and spontaneous eryptosis as well as susceptibility to Bay 11-7082 and parthenolide highest in aged erythrocytes. Thus, inhibition of NFκB signalling alone is not responsible for the stimulation of eryptosis by parthenolide or Bay 11-7082.

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Introduction

Senescence of nucleated cells is defined as an irreversible cellular growth arrest and is induced by internal factors, such as oncogenes [1], or external stimuli, e. g. cytokines [2]. Anucleated, circulating human erythrocytes are unable to proliferate and thus are growth-arrested per se. As a consequence, their life span is limited by a special form of senescence leading to a gradual process of cellular shrinkage and phosphatidylserine exposure, and eventually to clearance of aged erythrocytes after approx. 120 days [3]. Mechanisms accounting for erythrocyte senescence include a conformational change of the band 3 membrane protein domain thus leading to the appearance of an antigen specific for senescent cells. This triggers binding of specific autologous immunoglobulin G and subsequent removal of senescent erythrocytes by macrophages, such as Kupffer cells in the liver [4]. The conformational change presumably results from oxidative damage of membrane lipids, membrane proteins, or hemoglobin [5-9].

Senescent erythrocytes have been shown to be particularly sensitive to triggers of eryptosis [10], the suicidal erythrocyte death characterized by breakdown of cell membrane integrity, phosphatidylserine asymmetry and cell shrinkage [11]. Eryptosis is triggered by increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [11]. Ca^{2+} may enter erythrocytes through Ca^{2+}-permeable cation channels [12, 13], which are activated by oxidative stress [14]. Increased $[Ca^{2+}]_i$ activates Ca^{2+}-sensitive K^+ channels [15] and subsequently K^+ exit, hyperpolarization, Cl^- exit and water loss that leads to cell shrinkage [16]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface [17]. Ca^{2+} sensitivity of cell membrane scrambling is enhanced by ceramide [18]. Eryptosis is further stimulated by energy depletion [19] and caspase activation [20-24]. Signalling of eryptosis involves several kinases including AMP activated kinase (AMPK) [13], cGMP-dependent protein kinase [25], Janus-activated kinase 3 (JAK3) [26], casein kinase [27, 28], p38 kinase [29], PAK2 kinase [30] as well as sorafenib- [31] and sunifinib- [32] sensitive kinases.

Eryptosis is observed in several clinical conditions [11], such as diabetes [24, 33, 34], renal insufficiency [35], hemolytic uremic syndrome [36], sepsis [37], malaria [38-43], sickle cell disease [44], Wilson's disease [42], iron deficiency [45], malignancy [46], phosphate depletion [47], and metabolic syndrome [48]. Moreover, eryptosis is triggered by a wide variety of xenobiotics [10, 32, 48-67] including Nuclear Factor κ B (NFκB) inhibitors Bay 11-7082 and parthenolide [68]. NFκB is known as a transcription factor fostering cell survival [69-71]. The possibility was therefore considered that NFκB participates in the signalling of eryptosis [72]. Alternatively, the inhibitors are in part effective by mechanisms unrelated to NFκB-inhibition.

The present study explored if the amount of NFκB changes during the erythrocyte's aging and if a correlation between enhanced eryptosis in aged erythrocytes [10] and NFκB abundance exists. To demonstrate the possible functional relationship between age-sensitive NFκB abundance and age-sensitive eryptosis, the effect of Bay 11-7082 and parthenolide have been tested in young and aged erythrocytes. As a result, a correlation was found between erythrocyte age and NFκB abundance on the one hand as well as eryptosis on the other. However, the fact that both NFκB inhibitors showed the highest eryptosis induction in aged erythrocytes, where NFκB abundance is lowest, indicates an NFκB-independent mode of action.

Materials and Methods

Erythrocytes, solutions and chemicals

As described earlier [10], erythrocytes were isolated from the blood of healthy volunteers by following the guidelines of the ethical commission of the Radboud University Nijmegen Medical Centre. Blood was

collected in EDTA, and erythrocytes were fractionated according to cell volume using elutriation followed by a fractionation according to cell density using discontinuous Percoll gradients as described earlier [73]. This yields 24 fractions, that were combined to five fractions (I to V); whereby fraction I comprises the youngest, and fraction V the oldest cells [73, 74]. These fractions have been characterized with respect to cell survival *in vivo*, hemoglobin content, metabolome characteristics and membrane composition. The mean corpuscular volume (MCV) of these fractions was measured using a haematology analyzer (Sysmex XT1800i, Sysmex Corporation, Kobe, Japan).

Fractionated 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 or 48 hours [10]. Bay 11-7082 (Sigma, München, Germany) or parthenolide (Biomol, Hamburg, Germany) were added to the Ringer solution at the indicated concentrations for 24 or 48 hours. For this, stock solutions of Bay 11-7082 or parthenolide in dimethyl sulfoxide (DMSO) were diluted in Ringer solution. DMSO did not exceed final concentrations of 0.1%. Vehicle-treated erythrocytes served as controls.

Phosphatidylserine exposure and forward scatter

Erythrocyte fractions I to V or the corresponding unfractionated population (or whole blood indicated as WB) were incubated in Ringer solution in the absence or presence of Bay 11-7082 or parthenolide. After incubation, erythrocytes were washed in annexin-binding buffer at pH 7.4 containing (in mM): 125 NaCl, 10 HEPES and 5 $CaCl_2$. Erythrocytes were then stained with Annexin-Fluos from Roche Diagnostics (Mannheim, Germany) at a 1:35 dilution and mixed gently on a vortex mixer. After 20 min incubation in the dark at room temperature, samples were diluted 1:5 with annexin-binding buffer, thoroughly mixed to achieve single cell suspensions, and analysed by flow cytometry on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cell volume differences were estimated by forward scatter (FSC), and annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm as described earlier [10].

Western blotting

To determine age-dependent differences in the expression of NFκBs (i.e. p50 and p65 subunits), human erythrocytes were separated into five fractions (fractions I to V). Each fraction (250 µl erythrocyte pellet containing approx. 1.0 x 10^9 cells) was lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) hypotonic shock solution containing 1"complete protease inhibitor cocktail" tablet from Roche Diagnostics. Ghost membranes were pelleted (15.000 x g, for 30 min at 4°C) and lysed in 250 µl lysis buffer pH 7.4 containing 125 mM NaCl, 25 mM HEPES, 10 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 0.4% β-mercaptoethanol and 1 "complete protease inhibitor cocktail" tablet. Lysed ghost membranes were solubilized in Laemmli sample buffer at 95°C for 5 min, resolved by 8% SDS-PAGE and electrophoretically transferred onto a PVDF membrane (Roth, Karlsruhe, Germany) as described earlier [68]. Membranes were then incubated in blocking solution (5% nonfat milk in tris-buffered saline (TBS) containing 0.01% Tween 20 (TBST)) at room temperature for 1 h. For detection of NFκB subunits p65 and p50, the membranes were incubated with a 1:1000 dilution of affinity purified rabbit anti-NFκB p65 or anti-NFκB p50 (Cell Signaling Technology Inc., Danvers, MA, USA) at 4°C overnight. After washing membranes with TBST, immunoreactive proteins were visualized using enhanced chemoluminescence following incubation with a 1:5000 dilution of the secondary donkey-anti-rabbit horse-radish-peroxidase (HRP)-conjugated antibody (GE Healthcare, München, Germany) for 1 h at room temperature. β-actin was used as loading control and its detection was evaluated by an affinity isolated rabbit anti-β-actin antibody (Sigma-Aldrich, Taufkirchen, Germany). Immunoreactive bands were quantified by videodensitometry, and the NFκB p65/ß-actin ratio or NFκB p50/β-actin ratio of the samples was calculated.

Statistics

Data are expressed as arithmetic or geometric means ± SEM. Statistical analysis was made using ANOVA with Tukey's test as post hoc test, or using student´s t test where appropriate. A p value < 0.05 was considered statistically significant.

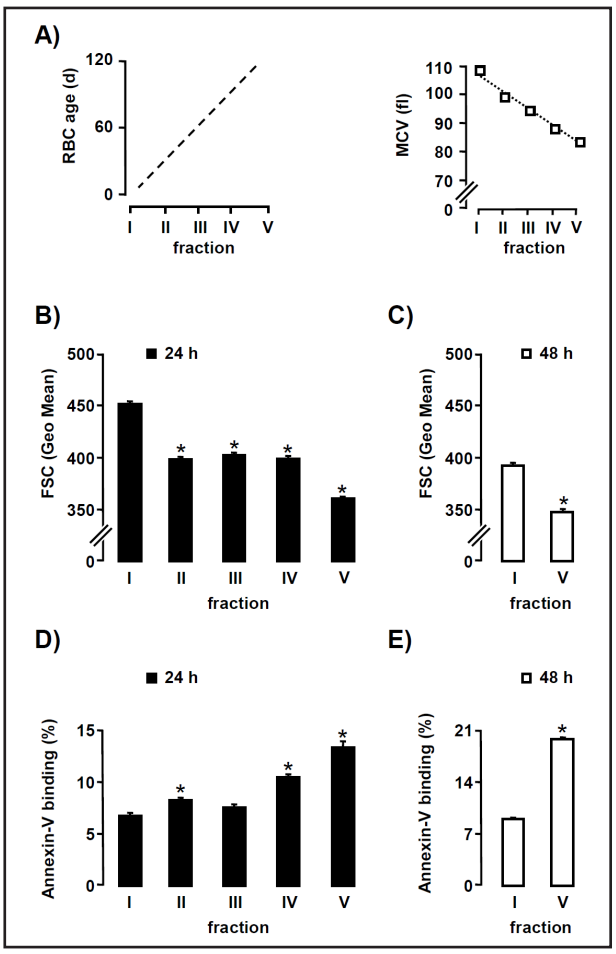

Fig. 1. Decreased erythrocyte volume and increased annexin-V binding in old erythrocytes. A. Means erythrocyte age (left) and mean corpuscular volume (MCV, right) in the five fractions isolated by volume and density fractionation. MCV data from one representative fractionation are shown. B. Means ± SEM (n = 3) of the erythrocyte forward scatter (geometric mean) of fractions I to V following incubation for 24 hours at 37°C in control Ringer solution. * significantly different from fraction I ($p < 0.05$; ANOVA). C. Means ± SEM (n = 3) of the erythrocyte forward scatter (geometric mean) of fractions I and V following incubation for 48 hours at 37°C in control Ringer solution. * significantly different from fraction I ($p < 0.05$; student´s t test). D. Mean percentage ± SEM (n = 3) of annexin V binding of erythrocytes from fractions I to V following incubation for 24 hours at 37°C in control Ringer solution. * significantly different from fraction I ($p < 0.05$, ANOVA). E. Mean percentage ± SEM (n = 3) of annexin V binding of erythrocytes from fractions I and V following incubation for 48 hours at 37°C in control Ringer solution. * significantly different from fraction I ($p < 0.05$; student´s t test).

Results

According to our previous study, the susceptibility to triggers of eryptosis is a function of erythrocyte age. Thus, erythrocytes have been separated into 5 fractions (I to V) according to age-associated differences in density and volume [10]. As illustrated in Fig. 1A, the MCV steadily declined with increasing age (higher fraction numbers) which served as a quality control for separation. In addition, cell volume decrease was confirmed by measurement of the forward scatter (FSC) which was lower in fraction V as compared with fraction I after incubation for 24 hours in Ringer solution (Fig. 1B). The forward scatter decreased even further following incubation in Ringer for 48 hours (Fig. 1C).

The decrease in forward scatter was paralleled by an increase of annexin V binding. As shown in Fig. 1D, after incubation for 24 hours in Ringer solution the percentage of annexin V binding erythrocytes was higher in fraction V than in fraction I. Incubation of the erythrocytes in Ringer solution for 48 hours resulted in a further increase of the percentage of annexin V binding cells (Fig. 1E).

In theory, the increased eryptosis of aged erythrocytes could be paralleled by changes of NFκB abundance. To explore the impact of erythrocyte age on the amount of NFκB, the levels of the NFκB subunits p65 and p50 have been determined by Western blotting. As shown in Fig. 2A, the abundance of the NFκB subunit p65 was highest in erythrocytes of fraction I. In erythrocytes of fraction V, the NFκB protein subunit p65 was virtually absent (Fig. 2A, C). Similar observations were made with NFκB subunit p50, which was again most abundant in fraction I and almost absent in fraction V (Fig. 2B, D).

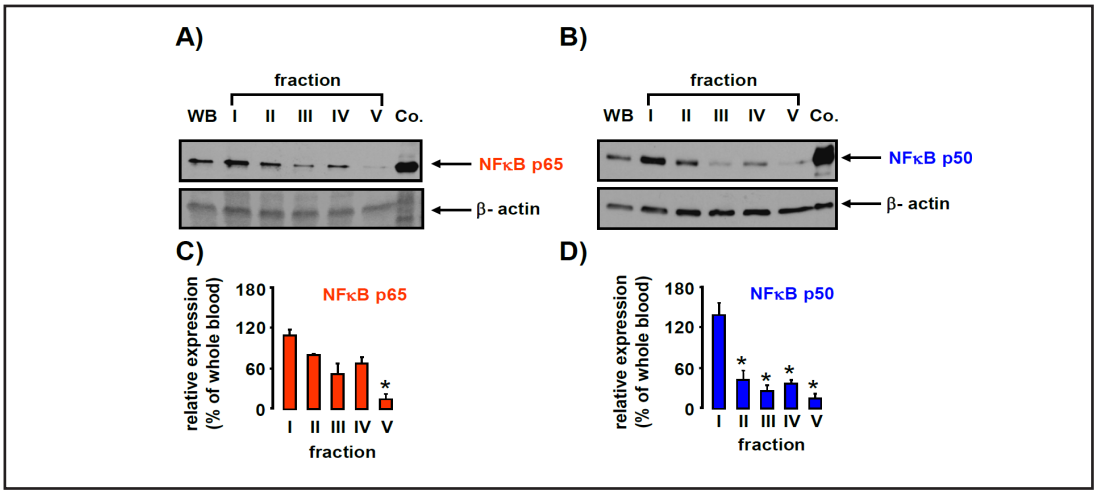

Fig. 2. Erythrocyte NFκB subunit abundance as a function of erythrocyte age. A. Western blot demonstrating the expression of NFκB protein subunit p65 (upper panel) and ß-actin (lower panel) in fractions I to V of a representative erythrocytes fractionation. WB = whole blood extracts (unfractionated); Co. = positive control for p65 expression. B. Western blot demonstrating the expression of NFκB protein subunit p50 (upper panel) and ß-actin (lower panel) in fractions I to V of a representative erythrocytes fractionation. WB = whole blood extracts (unfractionated); Co. = positive control for p50 expression. C. Arithmetic means ± SEM (n = 3) of the NFκB protein subunit p65/ß-actin abundance in erythrocytes of fractions I to V. Data are given as relative expression values (% of whole blood). * significantly different from fraction I ($p < 0.05$). D. Arithmetic means ± SEM (n = 3) of the NFκB protein subunit p50/ß-actin abundance in erythrocytes of fractions I to V. Data are given as relative expression values (% of whole blood). * significantly different from fraction I ($p < 0.05$).

Fig. 3. Positive correlation between erythrocyte NFκB subunit abundance and erythrocyte forward scatter. A. Erythrocyte forward scatter following incubation for 24 hours in control Ringer solution as a function of NFκB protein subunit p65/ß-actin abundance. B. Erythrocyte forward scatter following incubation for 24 hours in control Ringer solution as a function of NFκB protein subunit p50/ß-actin abundance.

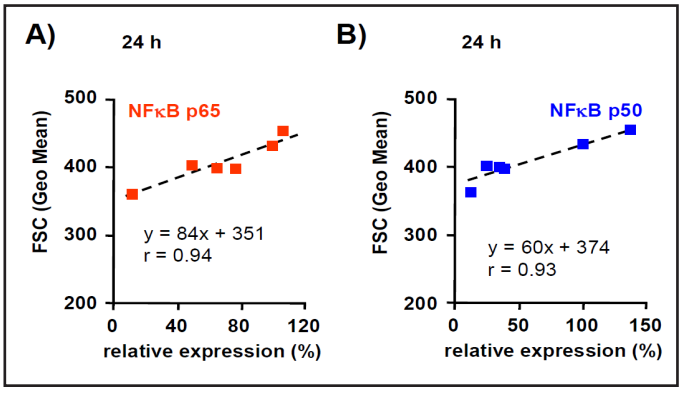

Fig. 3 displays the result of erythrocyte forward scatter after 24 h of incubation as a function NFκB subunit p65 (Fig. 3A) or NFκB subunit p50 (Fig. 3B) abundance. Fig. 4 displays the percentage of annexin V binding erythrocytes after a 48 hours incubation in Ringer solution as a function of NFκB subunit p65 (Fig. 4A) or NFκB subunit p50 (Fig. 4B) abundance.

The positive correlation between the forward scatter, and the negative correlation between the percentage of annexin V-exposing erythrocytes and the amount of NFκB subunits p50 and p65 does not necessarily reflect a causal relationship between the NFκB subunits abundance and the susceptibility to eryptosis. However, if such a causal relationship would exist, then pharmacological inhibition of the NFκB signalling pathway should trigger

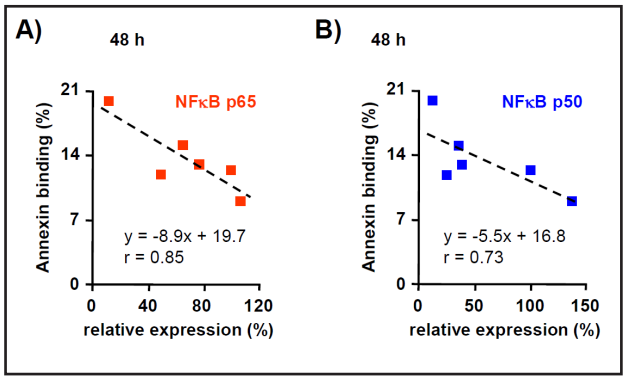

Fig. 4. Inverse correlation between erythrocyte NFκB subunit abundance and phosphatidylserine exposure. A. The percentage of Annexin V binding erythrocytes following incubation for 48 hours in control Ringer solution as a function of NFκB protein subunit p65/ß-actin abundance. B. The percentage of Annexin V binding erythrocytes following incubation for 48 hours in control Ringer solution as a function of NFκB protein subunit p50/ß-actin abundance.

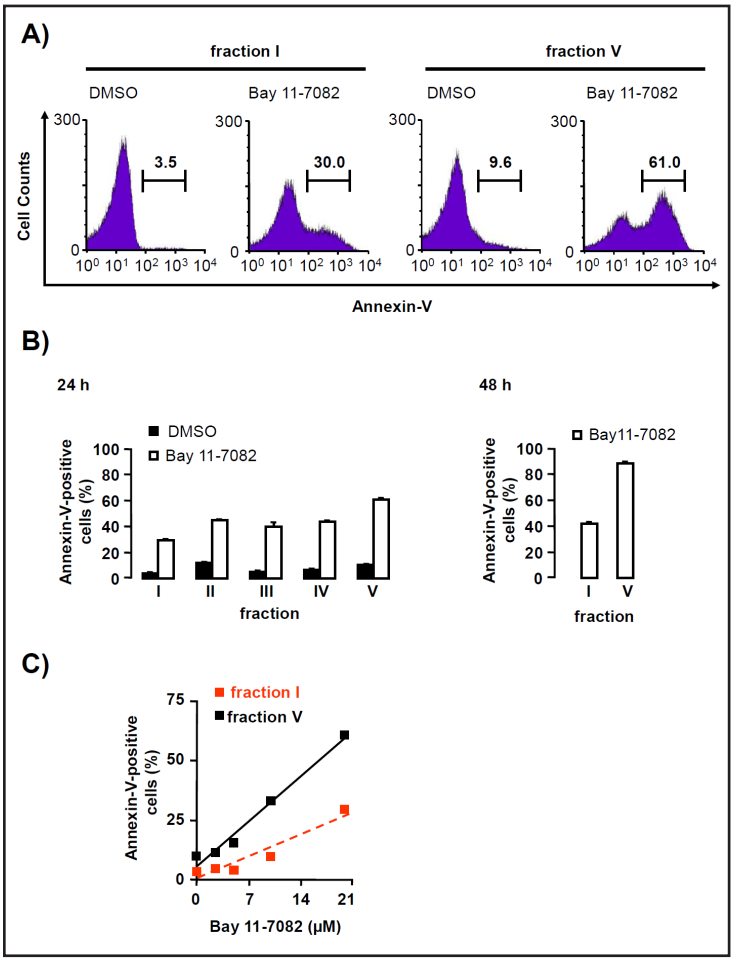

Fig. 5. Effect of Bay 11-7082 on phosphatidylserine exposure of erythrocytes of different age. A. Original histograms of annexin V binding erythrocytes in fraction I (left) or fraction V (right) following exposure for 24 h to Ringer solution in the absence (DMSO) or presence of 21 μM Bay 11-7082. B. Mean percentage ± SEM in fractions I to V (n = 3) of annexin V binding erythrocytes following exposure for 24 h (left) or 48 h (right) to Ringer solution in the absence (DMSO) or presence of 21 μM Bay 11-7082. Note that erythrocytes from fraction V are more susceptible to Bay 11-7082-induced eryptosis as compared with erythrocytes from fraction I. C. Annexin V binding of erythrocytes from fraction I (red squares) or fraction V (black squares) as a function of Bay 11-7082 concentration following exposure for 24 h. Data represent the mean of 3 determinations.

eryptosis. Moreover, pharmacological inhibition of NFκB signalling should dissipate the difference between young and aged erythrocytes, if mainly the lack of NFκB accounted for the enhanced susceptibility of aged erythrocytes to eryptosis. As illustrated in Fig. 5, the opposite was true. Treatment of erythrocytes with 20 μM Bay 11-7082, a concentration which should completely block NFκB, was followed by stimulation of annexin V binding, an effect more pronounced in aged erythrocytes than in young erythrocytes (Fig. 5A, B). Accordingly, titration of Bay 11-7082 did not dissipate but enhanced the difference of annexin V binding between erythrocytes of fraction I and erythrocytes of fraction V (Fig. 5C). Similar observations were made with 100 μM parthenolide (Fig. 6A, B), which again did not dissipate but enhanced the difference of annexin V binding between young and old erythrocytes (Fig.

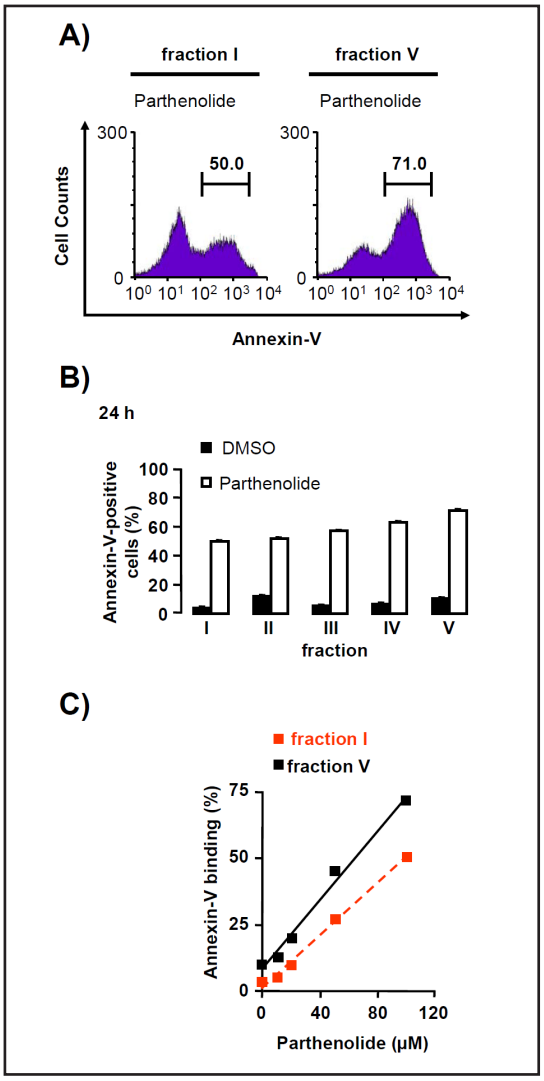

Fig. 6. Effect of parthenolide on phosphatidylserine exposure of erythrocytes of different age. A. Original histograms of annexin V binding erythrocytes in fraction I (left) or fraction V (right) following exposure for 24 h to Ringer solution in the presence of 100 μM parthenolide. B. Mean percentage ± SEM in fractions I to V (n = 3) of annexin V binding erythrocytes following exposure for 24 h to Ringer solution in the absence (DMSO) or presence of 100 μM parthenolide. Note that erythrocytes from fraction V are more susceptible to parthenolide-induced eryptosis as compared with erythrocytes from fraction I. DMSO controls are the same as shown in Fig. 5B. C. Annexin V binding of erythrocytes from fraction I (red squares) or fraction V (black squares) as a function of parthenolide concentration following exposure for 24 h. Data represent the mean of 3 determinations.

6C). Thus, in aged erythrocytes with the lowest NFκB abundance, the inverse correlation between the higher sensitivity to parthenolide and Bay 11-7082-induced eryptosis and the NFκB expression level has to be based on NFκB-independent modes of action of the inhibitors. As a consequence, young erythrocytes, with their high NFκB abundance and the associated low sensitivity to parthenolide- and Bay 11-7082-induced eryptosis, require a higher concentration of parthenolide or Bay 11-7082 in order to achieve the same rate of eryptosis as aged erythrocytes (Fig. 6C and Fig. 5C, respectively).

Discussion

Our data confirm the previous observation that aged erythrocytes are particularly sensitive to eryptosis [10]. The present study further reveals that the amount of NFκB is constantly decreasing during an erythrocyte's life span and negligible in aged erythrocytes. In view of the earlier observation that pharmacological inhibition of NFκB triggered eryptosis [68], the coincidence of low NFκB protein subunit abundance and high susceptibility to eryptosis could have reflected a causal relationship. If so, pharmacological inhibition of NFκB should be more effective in erythrocytes expressing high NFκB levels than in erythrocytes expressing low levels of NFκB. Accordingly, pharmacological inhibition of NFκB should dissipate the difference between young and aged erythrocytes. As illustrated in Fig. 5 and Fig. 6, the effects

of parthenolide or Bay 11-7082 on eryptosis were more pronounced in aged than in young erythrocytes. Accordingly, the inhibitors augmented the differences between erythrocytes of fraction I and erythrocytes from fraction V. Thus, the difference in susceptibility to inhibitor-induced eryptosis between young and aged erythrocytes was not due to the differences in NFκB abundance. Moreover, even though the substances are expected to inhibit NFκB, their effectivity in erythrocytes of fraction V, i.e. in the virtual absence of NFκB, indicates that the substances trigger eryptosis by mechanisms other than inhibition of NFκB. For example, these mechanisms could be glutathione (GSH) depletion (data not shown) [68, 75-77] and/or caspase activation [78-80]. Parthenolide with its properties to inhibit many components of the canonical NFκB signalling pathway, e.g. IKKs [81-83], and NFκB p65 [84-86] seems to be a much better candidate to investigate the possible role of NFκB signalling in cell death mechanisms in erythrocytes than Bay 11-7082 which in this pathway solely inhibits IKK [87-89]. Bay 11-7082 was originally identified as an inhibitor of the NFκB signalling pathway [90].

We have shown previously that old erythrocytes contain higher Ca^{2+} levels [10]. Thus, age-sensitive eryptosis is partially Ca^{2+}-dependent, e.g. through Ca^{2+}-mediated activation of the scramblase and the Gardos channel. The analysis of the relationship between Redox-sensitive NFκB activity and Ca^{2+}-induced cell death, as demonstrated in nucleated cells [91], will be a challenging task of future eryptosis research.

The enhanced *in vitro* susceptibility of aged erythrocytes towards parthenolide should lead to their elimination, and thus to a drift towards younger erythrocyte populations *in vivo*. In consequence, this could enhance erythropoiesis resulting in increased reticulocyte counts *in vivo*. However, clinical studies using feverfew extracts or parthenolide did not show any significant toxicity [92]. Administration of the NFκB inhibitor ethacrynic acid [93] favourably influences the clinical course of sickle cell anemia by tight covalent binding of this compound to hemoglobin S [94]. Whether or not other NFκB inhibitors, e.g. parthenolide or Bay 11-7082, equally possess an anti-anemia effect or influence the numbers of circulating reticulocytes remains to be shown.

The present results do not address the potential role of NFκB for the regulation of gene expression in erythrocyte progenitor cells. In this context, evidence has been provided that the glucocorticoid receptor is needed for stress erythropoiesis [95]. As glucocorticoid receptors are known to inhibit NFκB transcriptional activity by direct physical interaction in a DNA-independent matter (for review see: [96]), stimulation of eryptosis by NFκB inhibitors might also be related to decreased abundance of glucocorticoid receptors in aged human erythrocytes. In addition, acting as transcription factors in progenitor cells, NFκBs may indeed control the expression of genes relevant for the susceptibility to eryptosis. Mature, circulating erythrocytes are, however, devoid of nuclei and unable to express novel proteins. In those cells, the NFκB protein abundance may be irrelevant for the susceptibility to eryptosis.

Interestingly, we can find certain parallels regarding the inverse correlation between NFκB abundance and eryptosis when we look at the proliferative rate of cancer cells and their NFκB activity. Cancer cells with low NFκB DNA-binding activity also exhibit a significantly higher sensitivity to the anti-proliferative effects of parthenolide and vice versa [97], i.e. cancer cells with a high NFκB DNA-binding activity show a significantly lower sensitivity to the anti-proliferative effects of parthenolide.

In conclusion, our current study clearly indicates an inverse correlation between erythrocyte age and NFκB abundance. Thus, NFκB protein was most abundant in young erythrocytes and virtually absent in aged erythrocytes. Concomittantly, NFκB inhibitor-induced eryptosis was most pronounced in aged erythrocytes, pointing to NFκB-independent mechanisms leading to an enhanced susceptibility of aged erythrocytes to parthenolide- and Bay11-7082-induced eryptosis.

Conflict of Interest

Competing interests: the authors have no competing interests.

Acknowledgements

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