Stimulation of Suicidal Erythrocyte Death by Lipoxygenase Inhibitor Bay-Y5884

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Key Words

Cell volume • Lipoxygenase • Cation channels • Calcium • Apoptosis • PGE₂

Abstract

The prostaglandin PGE₂, a metabolite of the cyclooxygenase pathway, activates Ca²⁺-permeable cation channels in erythrocyte cell membranes leading to entry of Ca²⁺ with subsequent eryptosis, i.e. cell shrinkage, breakdown of phosphatidylserine (PS) asymmetry and membrane blebbing, all features typical for apoptosis in nucleated cells. PS exposing cells are recognized by macrophages, engulfed, degraded and thus cleared from circulating blood. The present study explored whether the specific lipoxygenase inhibitor Bay-Y5884 influences eryptosis. As determined by competitive ELISA, Bay-Y5884 (20 µM) enhanced the release of PGE₂ from human erythrocytes. According to whole-cell patch-clamp, Bay-Y5884 (20 µM) activated nonselective cation channels. The effect of Bay-Y5884 on cation channels was abolished by the cyclooxygenase inhibitor diclophenac (10 µM). Bay-Y5884 (30-40 µM) significantly increased erythrocyte free Ca²⁺ concentration and PS exposure as analyzed in flow cytometry by Fluo3 fluorescence and

annexin-V binding, respectively. PS exposure triggered by 20 µM (but not by 40 µM) Bay-Y5884 was blunted by cyclooxygenase inhibitors acetylsalicylic acid (50 µM) and diclophenac (10 µM). In conclusion, the lipoxygenase inhibitor Bay-Y5884 enhances erythrocyte PGE₂ formation with subsequent activation of non-selective cation channels, Ca²⁺ entry and phospholipid scrambling.

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Introduction

Erythrocytes have earlier been considered unable to undergo apoptosis, as they lack mitochondria and nuclei, key organelles in the apoptotic machinery of nucleated cells [1]. However, treatment of erythrocytes with the Ca²⁺-ionophore ionomycin has been shown to trigger membrane blebbing and break down of phosphatidylserine asymmetry of the cell membrane [2-5], typical features of apoptosis in other cell types [1, 6]. The breakdown of phosphatidylserine asymmetry results from scrambling of

the cell membrane, which is stimulated by increase of cytosolic Ca^{2+} activity [7, 8]. As macrophages are equipped with receptors specific for phosphatidylserine [9-11], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [12, 13]. Thus, increase of cytosolic Ca^{2+} activity fosters the clearance of affected erythrocytes. Ca^{2+} further activates Ca^{2+} sensitive K^{+} channels [14, 15], fostering cell shrinkage [16], another typical feature of apoptotic cells [Lang et al. 1998]. The similarity of suicidal erythrocyte death to the apoptosis of nucleated cells prompted the term eryptosis for the description of this particular erythrocyte death [17]. The molecular mechanisms of eryptosis are similar but not necessarily identical to those leading to erythrocyte senescence [17-21] or neocytolysis [20].

Stimulators of Ca^{2+} entry include hyperosmotic shock by increase of extracellular osmolarity, oxidative stress by addition of *t*-butylhydroperoxide (*t*-BHP) and energy depletion by removal of extracellular glucose [5], which all activate Ca^{2+} permeable cation channels [22, 23].

Most recent observations revealed that those channels are activated by PGE_2 [24]. The effects of hyperosmotic shock and removal of extracellular Cl^{-} on cation channel activity and phosphatidylserine exposure were paralleled by release of PGE_2 , mimicked by exogenous addition of PGE_2 and blunted in the presence of cyclooxygenase inhibitors [24]. It was thus concluded that formation of PGE_2 and subsequent activation of cation channels contributed to the stimulation of erythrocyte "apoptosis" following hyperosmotic shock and Cl^{-} removal.

The present experiments have been performed to explore whether other eicosanoid signaling pathways are involved in the regulation of eryptosis. As evidence for lipoxygenase activity in erythrocytes has been obtained previously [25-28], the effect of the specific lipoxygenase inhibitor Bay-Y5884 has been explored. It is shown that Bay-Y5884 enhances erythrocyte PGE_2 release, activates cation channels, leads to Ca^{2+} entry and triggers erythrocyte phosphatidylserine exposure.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers and used either without purification or after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/

Fig. 1. Bay-Y5884-induced formation of PGE_2 . Stimulation of PGE_2 release by exposure to Bay-Y5884 (20 μM , open squares). Shown is the PGE_2 accumulation in the supernatant during exposure of human erythrocytes to Bay-Y5884 as determined by competitive immunoassay. Data are means $\pm$ SEM; $n = 3-5$ and given as % of controls (106 ± 5 pg per 10^9 cells). For controls, erythrocytes were incubated for the respective time periods in drug free normal NaCl Ringer medium.* indicates significant difference from the respective control values ($P = 0.05$, ANOVA).

NaOH, 5 glucose, 1 CaCl_2 ; pH 7.4. For chloride-free conditions, a 0 Cl^{-} bath solution was used containing 125 Na-gluconate, 32 HEPES/NaOH (pH 7.4), 5 glucose, 1 $\text{Ca}(\text{gluconate})_2$, and 1 MgSO_4 . Where indicated, lipoxygenase inhibitor Bay-Y5884 (20-40 μM), cyclooxygenase inhibitors acetylsalicylic acid (50 μM) or diclophenac (10 μM), were added. The substances were dissolved in final concentrations of 0.1 % dimethyl sulfoxide (DMSO) (Bay-Y5884, ionomycin and diclophenac), or in water (acetylsalicylic acid). Acetylsalicylic acid, diclophenac and ionomycin were purchased from Sigma (Taufkirchen, Germany), Bay-Y5884 was from BAYER (Leverkusen, Germany).

Determination of PGE_2

One billion erythrocytes were treated with 0.9 ml NaCl Ringer solution (control) with or without addition of Bay-Y5884 (20 μM). After incubation, cells were pelleted by centrifugation at 4 °C, 450 x g for 5 min. The supernatant was removed and stored at -20°C. The cells were lysed by addition of 0.4 ml 10 mM HEPES (pH 7.4). The lysate was cleared by centrifugation at 4°C, 22000 g for 10 min and likewise stored at -20°C. PGE_2 concentrations in the supernatant and in the lysate were determined using the Correlate-EIATM Prostaglandin E_2 Enzyme Immunoassay Kit from Assay Designs, Inc. (Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, the samples were diluted 1:2.5 with assay buffer. Then, 100 μl sample, 50 μl alkaline phosphatase PGE_2 conjugate and 50 μl monoclonal

Fig. 2. Bay-Y5884 induced activation of nonselective cation conductance. **A.** Whole-cell current traces from a human erythrocyte recorded successively with Na-gluconate (Cl-free) bath solutions before (left) and after bath application of 20 μ M Bay-Y5884 (middle) or 20 μ M Bay-Y5884 together with 10 μ M diclophenac (right). The pipette contained Na-gluconate solution. The dotted line indicates the zero current value. Membrane voltage was held at -20 mV. **B.** Mean current-voltage relationships ($\pm$ SEM; $n = 6$, each) of human erythrocytes recorded in paired experiments with Na-gluconate pipette solution and either NaCl containing (open circles) or Cl-free (Na-gluconate, closed symbols) bath solutions in the absence (closed circles) or in the presence of Bay-Y5884 (20 μ M, closed triangles). **C.** Mean whole-cell conductance ($\pm$ SEM; $n = 6$, each) as recorded in (B) with NaCl, Na-gluconate, Na-gluconate/Bay-Y5884 and Na-gluconate/Bay-Y5884/diclophenac in the bath. Data were calculated from (B) by linear regression. * indicates significant difference ($p < 0.05$; ANOVA).

anti-PGE₂ EIA antibody were applied to goat anti-mouse IgG microtiter plates and incubated at room temperature for 2 h. After washing, 200 μ l p-nitrophenyl phosphate substrate solution was added and incubated at room temperature for 45 min. Finally, the optical density at 405 nm was measured in a microplate reader. PGE₂ concentrations in the samples were calculated from a PGE₂ standard curve (39.1 – 5000 pg/ml) which was run in parallel. PGE₂ levels in the supernatant of control erythrocytes were 106 ± 5 pg per 10^9 cells, and were set as 100 %. PGE₂ levels in the samples of erythrocytes exposed to Bay-Y5884 were calculated as % of the appropriate controls.

Patch clamp

Patch clamp experiments have been performed at room temperature in voltage-clamp, fast-whole-cell mode according to Hamill et al. [29]. The cells were continuously superfused

through a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution (see above). Borosilicate glass pipettes (8–12 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Whole-cell currents were determined at ten successive 700-ms square pulses from the -20 mV holding potential to potentials between -100 mV and +80 mV. The current values were 3 kHz low-pass filtered.

The pipette solution contained (in mM): 115 Na-gluconate, 10 NaCl, 1 MgATP, 1 EGTA, and 5 HEPES/NaOH (pH 7.4) and

Fig. 3. Bay-Y5884-induced increase of erythrocyte free Ca^{2+} concentration. A. Representative FACS histograms showing the Ca^{2+} -sensitive fluorescence of Fluo3 loaded erythrocytes incubated for 3 h either in NaCl Ringer solution (left) or in NaCl Ringer containing Bay-Y5884 (40 μM ; right). B. Mean ($\pm$ SEM) fluorescence of erythrocytes incubated for different time periods in the absence (control) or presence of Bay-Y5884 (40 μM , $n=12-13$); $(^{**})$ indicates significant difference to control; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, unpaired two-tailed t-test.

was used in combination with NaCl and Cl-free Ringer solutions (see above).

The offset potentials between both electrodes were zeroed before sealing. The potentials were corrected for liquid junction potentials as estimated according to Barry & Lynch [30]. The original whole-cell current traces are depicted without filtering (acquisition frequency of 5 kHz) and currents of the individual voltage square pulses are superimposed. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

FACS analysis

FACS analysis was performed essentially as described [31]. After incubation, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES; (pH 7.4) and 5 CaCl_2 . Erythrocytes were stained with Annexin-Fluos (Roche Diagnostics, Mannheim, Germany) at a 1:100 dilution. After 15 min, samples were diluted 1:5 and measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson, Heidelberg, Germany). Cells were analysed by forward and sideward scatter and annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} measurements were performed as described [32]. Briefly, erythrocytes were loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) by addition of 10 μl of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 10 ml erythrocyte suspension (0.16 % hematocrit in Ringer). The cells were incubated at 37°C for 15 min under vigorous shaking and protection from light. An additional 10 μl of Fluo-3/AM was added, with incubation carried out for 25 min. Fluo-3-AM-loaded erythrocytes were centrifuged at 1000 g for 3 min at 22 °C and washed two times with Ringer solution containing 0.5 % bovine serum albumin (Sigma) and once with Ringer. For flow cytometry, Fluo-3/AM-loaded erythrocytes were resuspended in 5 ml Ringer (0.32 % hematocrit) containing Bay-Y5884 (20 - 40 μM), the Ca^{2+} ionophore ionomycin (1 μM ; Sigma; Taufkirchen, Germany) or vehicle alone and incubated for different time periods at 37°C. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data were expressed as means $\pm$ SEM and statistical analysis was made by ANOVA or unpaired two-tailed t-test, as appropriate, $p < 0.05$ was considered statistically significant.

Fig. 4. Bay-Y5884-induced phosphatidylserine exposure. **A.** Representative FACS histograms depicting the annexin V fluorescence of erythrocytes (as a measure of phosphatidylserine exposure) incubated for 24 h in NaCl Ringer (control; upper panel, left) or in NaCl Ringer solution containing Bay-Y5884 (20 μ M) in the absence (upper panel, right) or in the presence of diclophenac (10 μ M, lower panel, left) or acetylsalicylic acid (aspirin, 50 μ M, lower panel, right). **B.** Concentration dependence of the Bay-Y5884 ($n = 9-18$) effect on erythrocyte annexin V binding (24 h incubation in normal Ca^{2+} -containing NaCl Ringer). Arithmetic means $\pm$ SEM. **C.** Inhibition of Bay-Y5884 induced annexin V binding by diclofenac (10 μ M) or acetylsalicylic acid (50 μ M). Mean percentage of annexin V binding erythrocytes ($\pm$ SEM; $n = 16-20$) stimulated as in (B) in the absence (open column) or presence (grey columns) of diclofenac or acetylsalicylic acid (aspirin). * indicates significant difference to Bay-Y5884 alone; $p = 0.05$; ### indicates significant difference to control (Bay-Y5884-free Ca^{2+} -containing NaCl Ringer); $p = 0.001$; ANOVA. **D.** Insensitivity to acetylsalicylic acid of annexin V binding in the presence of higher (40 μ M) Bay-Y5884 concentrations. Mean percentage of annexin V binding erythrocytes ($\pm$ SEM; $n = 14$) stimulated by Bay-Y5884 (40 μ M) as in (B) in the absence (open column) or presence (grey column) of acetylsalicylic acid (50 μ M, aspirin).

Fig. 5. Effect of Bay-Y5884 on forward scatter. A. Representative FACS histograms depicting the forward scatter of erythrocytes (as a measure of erythrocyte volume) incubated for 24 h in NaCl Ringer (control) or in NaCl Ringer solution containing Bay-Y5884 (30 μ M). B. Concentration dependence of the Bay-Y5884-induced effect on erythrocyte forward scatter (24 h incubation in normal Ringer; arithmetic means $\pm$ SEM, n = 6-10).

Results

As determined by competitive ELISA, a specific lipoxygenase inhibitor Bay-Y5884 (20 μ M) enhanced the release of prostaglandin PGE₂ (Fig. 1).

The formation of PGE₂ is expected to activate cation channels in the cell membrane [24]. Patch clamp experiments have thus been performed to explore the effect of the lipoxygenase inhibitor on the erythrocyte cation channel activity. The membrane conductance of human erythrocytes in NaCl Ringer solution was low, amounting to about 78 pS. Application of Na-gluconate bath solution resulted in an increase of the cation conductance, an effect which was indeed significantly enhanced by the lipoxygenase inhibitor Bay-Y5884 (20 μ M, Fig. 2). The effect of Bay-Y5884 on cation channel activity was abolished by the cyclooxygenase inhibitor diclophenac (10 μ M, Fig. 2A,C).

Activation of the cation channels should stimulate Ca²⁺ entry and thus increase cytosolic Ca²⁺ activity [5, 22, 23]. Thus, Fluo3 fluorescence was employed to test for an effect of the lipoxygenase inhibitor Bay-Y5884 on the erythrocyte cytosolic Ca²⁺ activity. As illustrated in Fig. 3, Bay-Y5884 (40 μ M) indeed increased the Fluo3 fluorescence.

An increase of cytosolic Ca²⁺ activity has previously been shown to trigger cell membrane scrambling leading to phosphatidylserine exposure at the cell surface [2-5]. The percentage of phosphatidylserine exposing erythrocytes has thus been determined. Annexin binding in flow cytometry was taken as a measure of phosphatidylserine exposure. As illustrated in Fig. 4A-C, Bay-Y5884 (20 μ M) significantly enhanced the percentage of annexin binding erythrocytes (13 $\pm$ 1 %, n=36). The effect of Bay-Y5884 (20 μ M) on annexin binding was significantly blunted by acetylsalicylic acid (50 μ M, Fig. 4A, C) or diclofenac (10 μ M, Fig. 4A, C). A higher concentration of Bay-Y5884 (40 μ M) dramatically enhanced the percentage of annexin binding erythrocytes (32 $\pm$ 4 %, n=18,

Fig. 4B and 4D). However, at this Bay-Y5884 concentration acetylsalicylic acid (50 μ M) could not block the effect of Bay-Y5884 (Fig. 4D).

Entry of Ca²⁺ is further expected to activate Ca²⁺ sensitive K⁺ channels and subsequent cell shrinkage [16], which should be apparent from a decrease of forward scatter in flow cytometry. As illustrated in Fig. 5, Bay-Y5884 (30 μ M) tended to decrease the forward scatter of erythrocytes, an effect, however, not reaching statistical significance.

Discussion

The present observations demonstrate that a specific lipoxygenase inhibitor Bay-Y5884 increases the formation of prostaglandin E₂ (PGE₂), activates unselective cation channels, increases cytosolic Ca²⁺ activity, and leads to cell membrane scrambling. The observations may point to a shunting of eicosanoids to the cyclooxygenase pathway following inhibition of lipoxygenase. The resulting, enhanced formation of PGE₂ could then trigger the cascade leading to phosphatidyl-

serine exposure. PGE₂ has previously been shown to trigger eryptosis at least in part by activating the cation channels and thus increasing cytosolic Ca²⁺ activity [24]. Furthermore, PGE₂ has been shown to be generated by mechanically stressed adult human red blood cells [33] and to activate nonselective cation channels [34].

For the triggering of eryptosis, however, concentrations of PGE₂ are required, which are presumably encountered only in pathophysiological conditions [34]. Clearly, inhibition of lipoxygenase does not generate a physiological condition. Accordingly, Bay-Y5884 may have additional effects not related to lipoxygenase inhibition. As a matter of fact, the incomplete efficacy of cyclooxygenase inhibition suggests that the substance may be effective through additional mechanisms, which may or may not be related to eicosanoid metabolism. Moreover, inhibition of cyclooxygenase blunts only the effect of 20 μM but not of 40 μM Bay-Y5884.

The phosphatidylserine exposure following treatment of the erythrocytes with Bay-Y5884 is at least partially secondary to Ca²⁺ sensitive cell membrane scrambling [2, 3]. Increased cytosolic Ca²⁺ is a main trigger for eryptosis. Known triggers of nucleated cell apoptosis, such as serum deprivation and staurosporine, do not affect survival of mature erythrocytes [4]. Ca²⁺ erythrocytes enters through amiloride sensitive, cell volume regulated cation channels which have been characterized earlier [5, 22, 23, 35].

Besides its influence on cell membrane scrambling, Ca²⁺ entry activates the Ca²⁺ sensitive K⁺ channel in the erythrocyte cell membrane, which leads to hyperpolarization of the cell membrane and subsequent erythrocyte loss of KCl [14, 15, 36], which is supported by the activity of Cl⁻ channels [37]. PGE₂ has indeed been shown to trigger the release of K⁺ from erythrocytes [38]. The following cellular release of K⁺ and Cl⁻ together with osmotically obliged water then leads to cell shrinkage [38]. Similar to what has been shown for nucleated cell apoptosis [39-45], cellular loss of K⁺ participates in the triggering of eryptosis [16].

The present observations may be relevant for the survival of erythrocytes. Phosphatidylserine exposing erythrocytes are prone to be taken up by macrophages. [12, 46] and are thus cleared from circulating blood [4]. Phosphatidylserine exposure may be also involved in erythrocyte ageing, which is likewise paralleled by an increase of the cytosolic Ca²⁺ activity [46, 47]. Moreover,

Ca²⁺ entry and subsequent phosphatidylserine exposure may result from oxidative stress or defects of antioxidative defence [48] and may contribute to the premature death of erythrocytes in certain erythrocyte disorders [49], such as sickle cell disease [50, 51], thalassemia [52] or iron deficiency [53, 54].

Eryptosis may further compromise microcirculation and erythrocytes may participate in the regulation of hemostasis [55-58].

Eryptosis may not only be influenced by eicosanoids generated in the erythrocytes themselves, but as well by lipid mediators released from other blood cells, from cells of the vascular wall or from extravascular tissue. For instance, PGE₂ could be released from platelets [38] or from renal tissue during acute renal failure, which is paralleled by trapping of erythrocytes in renal medulla [59, 60].

Eicosanoids and eicosanoid dependent regulation of cation channels and Ca²⁺ entry may not only affect eryptosis, but be similarly important for the regulation of nucleated cell survival. For instance, PGE₂ has been demonstrated to either stimulate [61] or inhibit [62-68] apoptosis of nucleated cells. The lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) has been shown to stimulate apoptosis of monocytic carcinosarcoma cells [69, 70], lymphocytic cells [71, 72], breast cancer cells [73] and pancreatic cancer cells [74], but to inhibit CD95 induced death of glioma cells [75] and cationic liposome-induced apoptosis of macrophages [76]. In none of those studies, however, the effect of lipoxygenase inhibitors on cation channel activity has been explored.

Acknowledgements

The authors acknowledge the technical assistance of E. Faber and K. Bauer, and the meticulous preparation of the manuscript by Lejla Subasic. This study was supported by the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3, La 315/6-1, La 315/13-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research) 01 KS 9602 and the Biomed program of the EU (BMH4-CT96-0602). E.S. has been supported by a grant of the Alexander von Humboldt Foundation, P.A.L. and D.S.K. were recipients of a stipend from the intramural ICCR program.

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