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PARTICIPATION OF LEUKOTRIENE C4 IN THE REGULATION OF SUICIDAL ERYTHROCYTE DEATH

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Eryptosis, the suicidal death of erythrocytes, is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Eryptosis is triggered by increase in cytosolic Ca^{2+} concentration upon energy depletion. The present study explored the involvement of leukotrienes. Western blotting was employed to detect the cysteinyl-leukotriene receptor cysLT1, competitive immune assay to determine leukotriene release from erythrocytes, Fluo3 fluorescence to estimate cytosolic Ca^{2+} concentration, forward scatter to analyse cell volume and annexin V-binding to disclose phosphatidylserine exposure. As a result, erythrocytes expressed the leukotriene receptor CysLT1. Glucose depletion (24 hours) significantly increased the formation of the cysteinyl-leukotrienes $C_4/D_4/E_4$. Leukotriene C_4 (10 nM) increased Ca^{2+} entry, decreased forward scatter, activated caspases 3 and 8, and stimulated annexin V-binding. Glucose depletion similarly increased annexin V-binding, an effect significantly blunted in the presence of the leukotriene receptor antagonist cinalukast (1 μM) or the 5-lipoxygenase inhibitor BW B70C (1 μM). In conclusion, upon energy depletion erythrocytes form leukotrienes, which in turn activate cation channels, leading to Ca^{2+} entry, cell shrinkage and cell membrane scrambling. Cysteinyl-leukotrienes thus participate in the signaling of eryptosis during energy depletion.

Key words: *eryptosis, calcium, cysLT1, phosphatidylserine*

INTRODUCTION

Energy depletion triggers suicidal erythrocyte death or eryptosis (1), which is characterized by cell shrinkage and scrambling of the cell membrane with phosphatidylserine exposure at the cell surface (2). Cell shrinkage and cell membrane scrambling are at least in part secondary to activation of Ca^{2+}-permeable erythrocyte cation channels with subsequent Ca^{2+} entry (3-8). The channels are activated by prostaglandin E_2 which is increased upon cell shrinkage and thus participates in the stimulation of suicidal erythrocyte death following osmotic shock (9). However, the eryptosis following energy depletion is not abrogated in the presence of cycloxygenase inhibitors (unpublished observations). Thus, additional mechanisms must contribute to the signaling of eryptosis following energy depletion.

Candidate signaling molecules are cysteinyl-leukotrienes C_4, D_4 and E_4. Erythrocyte progenitor cells express the cysteinyl-leukotriene receptor cysLT1 (10-12) and mature erythrocytes are able to metabolize leukotrienes (13-15). Leukotrienes may signal through increase in cytosolic Ca^{2+} activity (12, 16-27). On the other hand, Ca^{2+} has been shown to stimulate phospholipase A_2 leading to formation of the leukotriene precursor arachidonic acid and leukotrienes (28-31).

The present study was performed to elucidate, whether cysteinyl-leukotrienes participate in the stimulation of Ca^{2+} entry and subsequent eryptosis of mature erythrocytes following glucose withdrawal. As a first step, Western blotting was performed to determine expression of cysLT1. In a second step, the formation of cysteinyl-leukotrienes C4, D4 and E4 was determined in the presence and absence of glucose. In a third step the effects of cysteinyl-leukotriene C_4 on cytosolic Ca^{2+} activity, cell volume, caspase 3 and 8 activity, and cell membrane scrambling were explored. In a final step, the effects of the cysLT1 antagonist cinalukast (32) or of inhibition of the 5-lipoxygenase (33) using the inhibitor BW B70C on eryptosis following energy depletion were determined.

MATERIALS AND METHODS

Erythrocytes, solutions, and chemicals

Experiments were performed at 37°C with banked erythrocyte concentrates provided by the blood bank of the University of Tubingen (34). According to legislative standards in Germany, 1 μl of the erythrocyte concentrate could have contained at most 150 leukocytes compared to 10^7 erythrocytes. In erythrocyte concentrates leukocytes are, therefore, depleted by a factor of at least 2000 as compared to whole blood. The study was approved by the ethics committee of the University of Tubingen (184/2003V).

Ringer solution contained (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.

Leukotriene B4 and C4, the CysLT1 antagonist cinalukast, and the 5-lipoxygenase inhibitor BW B70C were purchased from Sigma (Schnelldorf, Germany). Leukotriene C4 was stored under argon gas to prevent degradation. Leukotriene B4 was

dissolved in ethanol, leukotriene C4 in methanol. The effect of the solvent methanol was investigated as follows: In the experiments shown in *Fig. 3A,B, 4A,B*, and *5A,B,C,D,E,F* the control samples contained the same amount of methanol as the samples treated with the highest concentration of Leuktriene C_4 shown in the respective figure.

FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described (35, 36). After incubation, cells were washed in Ringer solution containing 5 mM $CaCl_2$. Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured 48 hours after incubation as described previously (9, 37, 38). Briefly, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1. To study leukotriene C_4-induced Ca^{2+} uptake of erythrocytes at physiologically low concentrations, erythrocytes were stained with Fluo3 in Ringer containing 5 mM Ca^{2+} as decribed above. Then, erythrocytes were further handled in a box filled with argon gas to prevent leukotriene C_4 degradation. The samples were purged by argon gas for 2 minutes to remove oxygen. Subsequently, the samples were kept under argon gas for different time periods in the absence or presence of 10 nM leukotriene C4. Then, Fluo3-dependent fluorescence was determined as a measure of the cytosolic Ca^{2+} concentration as described above.

Determination of leukotriene formation

5×10^8 erythrocytes taken from erythrocyte concentrates were incubated in Ringer solution either with or without 5 mM glucose for 24 hours. After incubation, cells were pelleted by centrifugation at 4°C, 450 g for 5 min. The supernatants were removed and stored at -80°C. Leukotriene C_4,D_4,E_4 concentrations in the supernatant were determined using the Cysteinyl Leukotriene Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer`s instructions. Leukotriene release from erythrocytes is expressed as pg cysteinyl leukotriene determined in the supernatant per 10^9 erythrocytes. Despite the high depletion factor leukocyte contaminations could in theory account for leukotriene formation. To rule out this possibility, 50 µl fresh whole blood and erythrocytes were similarly exposed to 37°C, and leukotriene formation was detected. As a result, leukotriene formation in whole blood was at the most 10 times higher than in erythrocyte concentrates (data not shown). Thus, the contribution of the residual leukocytes in the erythrocyte concentrates was too low to significantly bias the result.

Immunoblotting

100 µl whole blood, buffy coat or concentrates of banked erythrocytes were washed in Ringer solution and then hypotonically lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors composed of 2.5 mM EDTA, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) from Roche (Mannheim, Germany) at 4°C. Membranes were pelleted (15,000 rpm for 20 min at 4°C). Then, membranes were solubilized in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton-X-100, 0.4% β-mercaptoethanol. The protein concentration of the samples was determined with the Bradford method (Biorad, Munchen, Germany) with bovine serum albumin (BSA; Sigma) as standard. Equal amounts of lysate protein (40 µg per lane) were separated by 10% SDS-PAGE, and proteins were transferred to a PVDF membrane. After blocking with 5% nonfat milk in TBS-0.1% Tween 20 at room temperature for 1 h, the blot was probed at 4°C overnight with a commercial rabbit CysLT1antibody (Gene Tex, Hiddenhausen, Germany; 1:500 dilution in TBS-0.1% Tween 20 - 5% nonfat milk). After washing in TBS-0.1% Tween 20, the blot was incubated with a secondary anti-rabbit antibody (1:2000 in TBS-0.1% Tween 20 - 5% nonfat milk) conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) for 1 h at room temperature. Antibody binding was detected with the enhanced chemoluminescence ECL kit (Amersham).

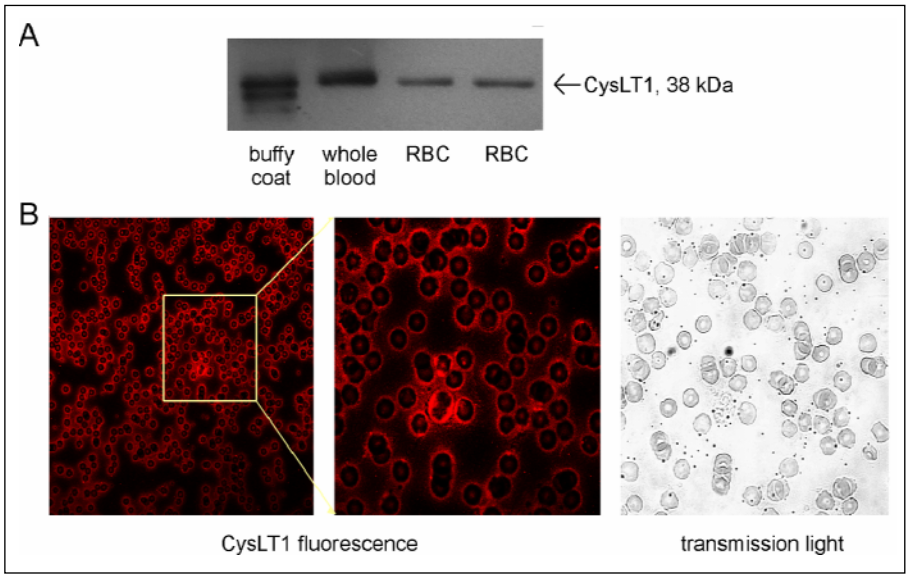

Fig. 1. Expression of the leukotriene receptor CysLT1 in human erythrocytes. **A**. Original Western Blot demonstrating the expression of CysLT1 in membrane preparations of enriched leukocytes (buffy coat), whole blood cells (whole blood) and human erythrocytes (RBC). **B**. Examination of CysLT1 expression in erythrocytes from a whole blood preparation by confocal microscopy. The left panels show CysLT1-dependent fluorescence in human erythrocytes. For comparison, the right panel shows the corresponding transmission light photograph.

Fig. 2. Effect of glucose depletion on leukotriene formation in human erythrocytes. Arithmetic means ± SEM (n = 4-5) of leukotriene $C_4/D_4/E_4$ abundance determined by competitive immunoassay in the supernatant following a 24 hours incubation of human erythrocytes at 37°C in glucose-containing (open bar) and glucose-deficient (closed bar) Ringer. * indicates significant difference from presence of glucose ($P < 0.05$, *t*-test).

Confocal microscopy

Fresh EDTA whole blood was taken and suspended in PBS at a cell density of $5*10^7$ cells/ml. 20 µl of the suspension were smeared onto a glass slide, air dried for 30 min, and then fixed with methanol for 2 min. After four washing steps with PBS for 10 min, the specimen was blocked by incubation with 10% goat serum. Following three washing steps with PBS for 5 min, the specimen was incubated with rabbit CysLT1 antibody (Gene Tex; 1:200) at 4°C overnight. The slide was washed again three times for 5 min and then incubated with Cy3-conjugated Affinipure goat anti-rabbit antibody (Jackson Immuno Research, Hamburg, Germany) at room temperature for 1.5 h. Then, the specimen was mounted using Prolong® Gold antifade reagent (Invitrogen, Karlsruhe, Germany). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC.

Caspase 3 and 8 assays

After incubation in the presence of LTC_4 or of vehicle alone, the activities of caspase 3 and 8 were determined independently using the CaspGlow Fluorescein Active Caspase-3 or -8 Staining kits from BioVision (Mountain View, CA, USA) according to the provided protocols.

Statistics

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by paired or unpaired t-test or ANOVA using Tukey's test as post hoc test, as appropriate.

RESULTS

The cysteinyl-leukotriene receptor CysLT1 is expressed in haematopoetic progenitor cells (10). Accordingly, we explored, whether the receptor is similarly expressed in mature erythrocytes. As illustrated in *Fig. 1A*, in erythrocyte membrane preparations a specific antibody directed against CysLT1 indeed bound to a protein band with the correct size (right lanes). The same band was readily detected in membrane preparations of whole blood cells containing leukocytes as well as in the membrane preparation of a buffy coat enriched in leukocytes. Further experiments were performed to determine CysLT1 expression in human erythrocytes

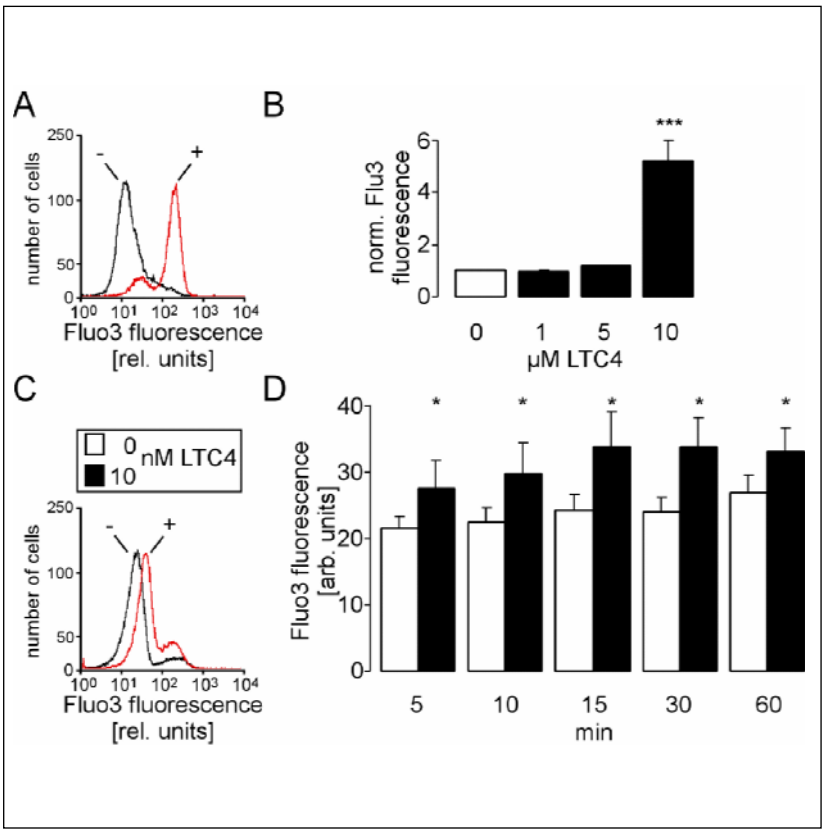

Fig. 3. Effects of Leukotriene C_4 on cytosolic Ca^{2+} concentration. **A**. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed to Ringer solution without (-) or with 10 µM leukotriene C_4 (+) for 48 hours at 37°C. **B**. Dose dependence of the effect of leukotriene C_4 on Fluo3 fluorescence. Arithmetic means ± SEM (n = 12-20) of the normalized geo means of Fluo3 fluorescence of erythrocytes exposed to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations for 48 hours at 37°C. *** ($P < 0.001$, ANOVA) indicates significant difference from values in the absence of leukotriene. **C**. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed to Ringer solution without (-) or with 10 nM leukotriene C_4 (+) in argon gas for 15 minutes. **D**. Arithmetic means ± SEM (n = 8-10) of Ca^{2+}-dependent Fluo3 fluorescence of erythrocytes incubated in argon gas to prevent leukotriene degradation at room temperature for the indicated time periods in the absence (open bars) or presence (closed bars) of 10 nM leukotriene C_4. * ($P < 0.05$, paired *t*-test) indicates significant difference from the absence of leukotriene C_4.

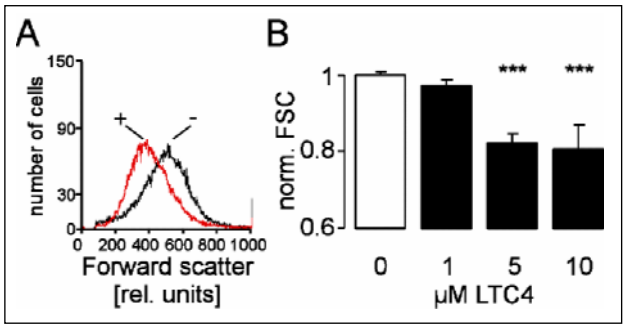

Fig. 4. Effects of Leukotriene C_4 on erythrocyte forward scatter. **A**. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed to Ringer solution without (-) or with 5 µM leukotriene C4 (+) for 48 hours. **B**. Dose dependence of the effect of leukotriene C_4 on forward scatter. Arithmetic means ± SEM (n = 12-16) of the normalized forward scatter of erythrocytes exposed to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations for 48 hours. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA).

using confocal microscopy. As shown in *Fig 1B*, confocal microscopy of a preparation of human whole blood indeed revealed the expression of CysLT1 on human erythrocytes.

In a next step, we explored the possibility that erythrocytes synthesize cysteinyl-leukotrienes. As shown in *Fig. 2*, a competitive immune assay indeed detected cysteinyl-leukotrienes in the medium (Ringer solution) of incubated erythrocytes. Moreover, the assay revealed that glucose depletion significantly increased the formation of cysteinyl-leukotrienes. Due to extreme instability of the different cysteinyl leukotrienes we could not discriminate between LTC_4, LTD_4 and LTE_4. All three leukotrienes are known to activate cysLT1 (12).

Erythrocytes express cation channels permeable to Ca^{2+} (39). Activation of those channels is expected to increase the cytosolic Ca^{2+} concentration. Therefore, Fluo3 fluorescence was employed to determine cytosolic Ca^{2+} activity in erythrocytes prior to and following treatment with different concentrations of cysteinyl-leukotriene LTC_4. As demonstrated in *Fig. 3A, B*, LTC_4-treatment was indeed followed by a significant increase in the cytosolic Ca^{2+} concentration. In contrast, leukotriene LTB4 did not significantly modify the cytosolic Ca^{2+} concentration (data not shown). LTC4 is known to be extremely instable. This might be the reason for the high concentrations of leukotrienes

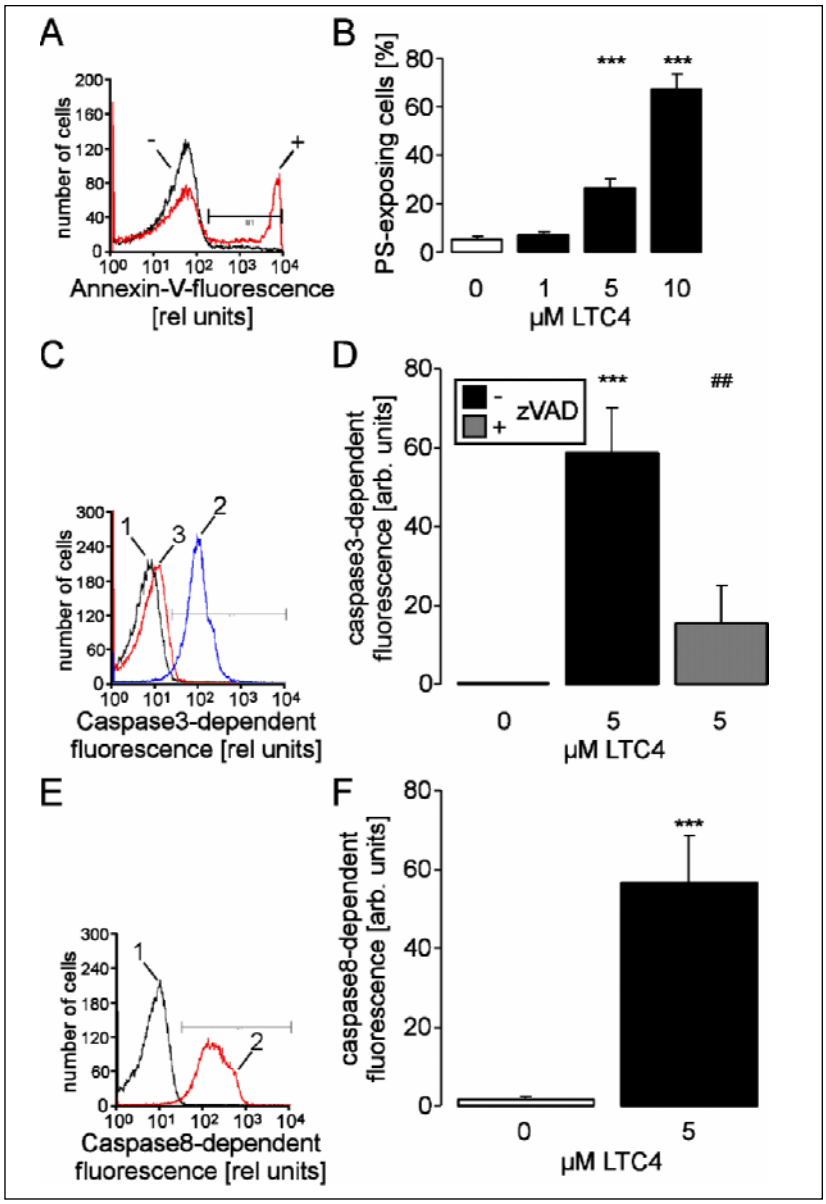

Fig. 5. Stimulation of phosphatidylserine exposure and caspase activation by Leukotriene C_4. **A**. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed to Ringer solution without (-) or with 5 µM leukotriene C_4 (+) for 48 hours. **B**. Dose dependence of the effect of leukotriene C_4 on phosphatidylserine exposure. Arithmetic means ± SEM (n = 12-20) of the percentage of annexin V-binding erythrocytes exposed to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations for 48 hours. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA). **C**. Histogram of caspase 3-dependent fluorescence of erythrocytes exposed for 48 hours to Ringer solution without (1) or with 5 µM leukotriene C_4 in the absence (2) or presence (3) of pancaspase inhibitor zVAD-FMK. **D**. Arithmetic means ± SEM (n = 10-12) of the percentage of erythrocytes with activated caspase 3 after exposure to Ringer solution without (white bar) or with 5 µM leukotriene C_4 in the absence (black bar) or presence of pancaspase inhibitor zVAD-FMK (grey bar) for 48 hours. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA). ## ($P < 0.01$) indicates significant difference from the absence of zVAD-FMK. **E**. Histogram of caspase 8-dependent fluorescence of erythrocytes exposed to Ringer solution without (1) or with 5 µM leukotriene C_4 (2) for 48 hours. **F**. Arithmetic means ± SEM (n = 12) of the percentage of erythrocytes with activated caspase 8 after exposure to Ringer solution without (white bar) or with 5 µM leukotriene C4 (black bar) for 48 hours. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (u-test).

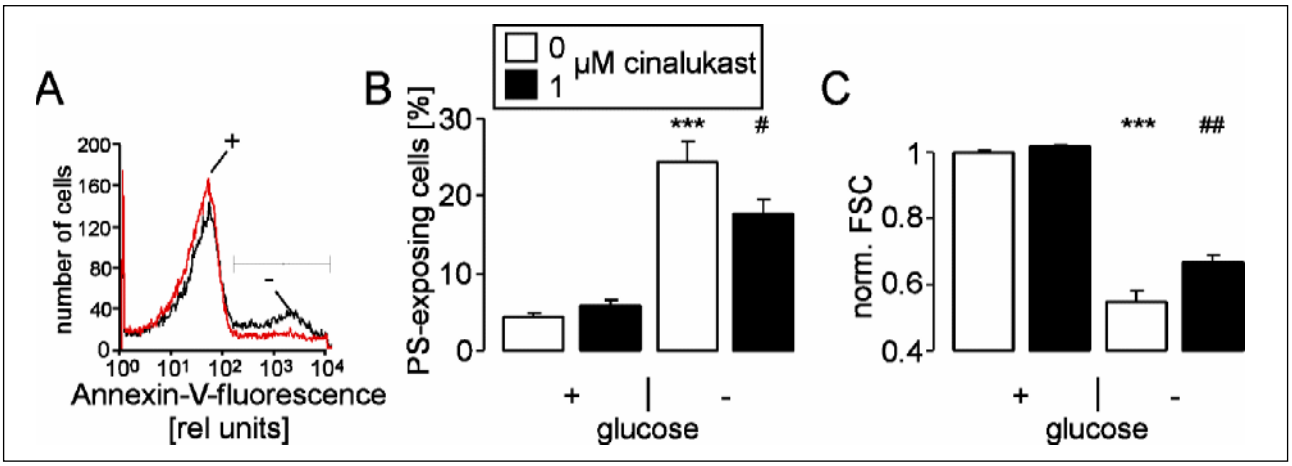

Fig.6. Inhibition of eryptosis by the CysLT1 antagonist cinalukast during energy depletion. **A**. Histogram of annexin V-binding in a representative experiment of erythrocytes incubated in Ringer solution free of glucose in the absence (-) or presence (+) of cinalukast (1 µM) for 48 hours. **B**. Arithmetic means ± SEM (n = 32) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of cinalukast (1 µM). *** indicates significant difference (ANOVA, P < 0.001) from control (presence of glucose). # indicates significant difference (ANOVA, P < 0.05) from absence of cinalukast. **C**. Arithmetic means ± SEM (n = 28) of normalized forward scatter of erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of cinalukast (1 µM). *** indicates significant difference (ANOVA, p<0.001) from control (presence of glucose). ## indicates significant difference (ANOVA, P < 0.01) from absence of cinalukast.

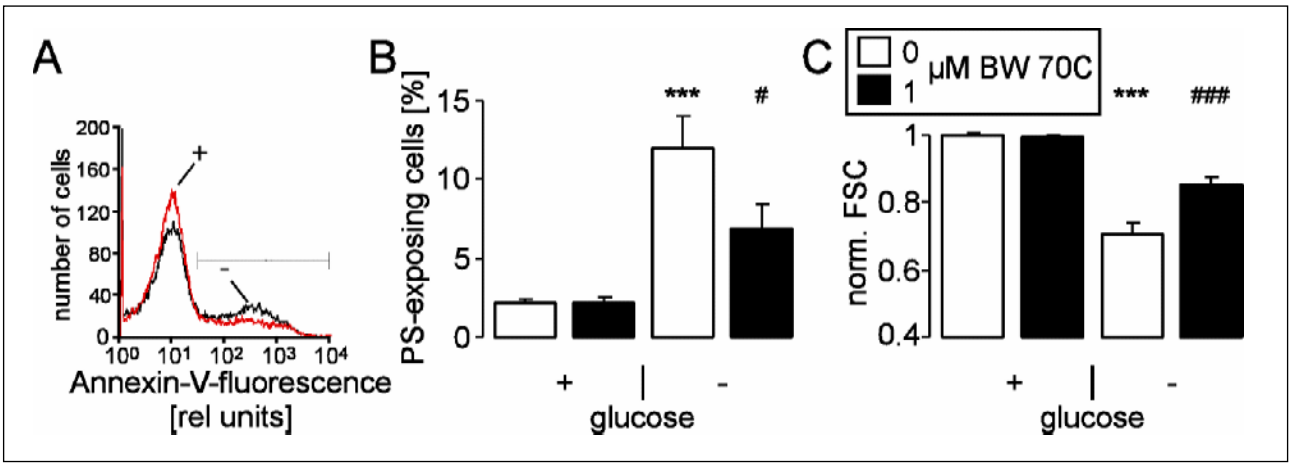

Fig.7. Inhibition of eryptosis by the 5-lipoxygenase inhibitor BW B70C during energy depletion. **A**. Histogram of annexin V-binding in a representative experiment of erythrocytes incubated in Ringer solution free of glucose in the absence (-) or presence (+) of BW B70C (1 µM) for 48 hours. **B**. Arithmetic means ± SEM (n = 19-20) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of BW B70C (1 µM). *** indicates significant difference (ANOVA, P < 0.001) from control (presence of glucose). # indicates significant difference (ANOVA, P < 0.05) from absence of BW B70C. **C**. Arithmetic means ± SEM (n = 19-20) of normalized forward scatter of erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of BW B70C (1 µM). *** indicates significant difference (ANOVA, p<0.001) from control (presence of glucose). ### indicates significant difference (ANOVA, P < 0.001) from absence of BW B70C.

required to induce Ca^{2+} influx into erythrocytes within 48 hours of incubation. To check this possibility, erythrocytes were exposed to 10 nM LTC4 in argon gas to prevent LTC4 degradation for different time periods, and Ca^{2+}-dependent Fluo3 fluorescence was monitored. As shown in *Fig. 3C, D*, 10 nM LTC4 induced a significant increase in the cytosolic Ca^{2+} concentration of erythrocytes within 5 min at room temperature.

An increase in the cytosolic Ca^{2+} concentration is expected to activate Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl and osmotically obliged water and thus to shrink the cells (40). Accordingly, the forward scatter was determined to depict alterations of cell volume. As shown in *Fig. 4*, LTC_4-treatment was indeed followed by a decrease of forward scatter.

An increase in the cytosolic Ca^{2+} concentration is further expected to trigger scrambling of the cell membrane with phosphatidylserine exposure at the cell surface (41, 42). Annexin V-binding to phosphatidylserine at the cell surface was utilized to detect cell membrane scrambling. As displayed in *Fig. 5A, B*, the treatment of erythrocytes with LTC_4 indeed significantly enhanced the percentage of annexin V-binding erythrocytes. LTB_4, applied at the same concentrations, did not induce appreciable annexin V-binding of erythrocytes (data not shown).

Further experiments revealed that LTC_4-induced eryptosis is associated with activation of caspases. As shown in *Fig. 5C, D*, LTC_4 led to activation of caspase 3, an effect, significantly blunted in the presence of the pancaspase inhibitor zVAD-FMK (*Fig. 5C, D*). Similarly, caspase 8 activity was significantly enhanced upon incubation in the presence of LTC_4 (*Fig. 5E, F*).

A further series of experiments explored, whether the activation of the cysteinyl-leukotriene receptor cysLT1 is involved in the stimulation of phosphatidylserine exposure and cell shrinkage during energy depletion as suggested from increased endogenous leukotriene formation during enery depletion (*Fig. 2*). As illustrated in *Fig. 6A, B*, the cysLT1 inhibitor cinalukast (1 μM) significantly blunted the effect of glucose withdrawal on cell membrane scrambling. Moreover, cinalukast significantly blunted erythrocyte shrinkage following glucose withdrawal as deduced from forward scatter (*Fig. 6C*).

If suicidal cell death of energy-depleted erythrocytes is indeed mediated by endogenously formed leukotrienes, inhibition of the 5-lipoxygenase should blunt phosphatdidylserine exposure and cell shrinkage following enery depletion. As shown in *Fig. 7A, B*, exposure of energy-depleted erythrocytes to 1 μM of the selective 5-lipoxygenase inhibitor BW B70C indeed significantly blunted phosphatidylserine exposure. Similarly, inhibition of the 5-lipoxygenase significantly reduced the cell shrinkage following energy depletion (*Fig. 7C*).

DISCUSSION

The present study discloses a novel function of leukotrienes, i.e. the stimulation of eryptosis. The cysteinyl-leukotriene C_4 presumably activates cation channels leading to influx of Ca^{2+}, cell shrinkage, and phosphatidylserine exposure at the erythrocyte surface. As glucose withdrawal enhances the formation of cysteinyl-leukotrienes, the effects participate in the signaling of eryptosis during energy depletion. Accordingly, antagonizing the cysteinyl-leukotriene receptor CysLT1 by cinalukast or inhibition of the 5-lipoxygenase by BW B70C blunt the eryptosis following glucose withdrawal and energy depletion.

While nothing has previously been known about leukotriene-induced increase in cytosolic Ca^{2+} activity and suicidal death of erythrocytes, several studies disclosed a role of leukotrienes in the regulation of cytosolic Ca^{2+} activity and cell survival of nucleated cells. Specifically, cysteinyl leukotrienes were shown to stimulate Ca^{2+} entry into mast cells (43). Interestingly, Ca^{2+} influx into mast cells was followed by stimulation of leukotriene formation (44, 45). Leukotrienes further increase cytosolic Ca^{2+} activity in human bronchiolar smooth muscle cells (46, 47), eosinophil leukocytes (48) and macrophages (49). Leukotrienes are further known to participate in volume regulation of nucleated cells (50). Ca^{2+} entry in turn is well known for its ability to trigger apoptosis (51). Several, partially conflicting observations demonstrate a role of leukotrienes in the regulation of cell survival (52-54). Cysteinyl leukotrienes are involved in the apoptotic effect of energy depletion in the brain (55), and leukotrienes have been shown to stimulate apoptosis (56). In contrast, leukotriene D4 has been shown to confer survival of intestinal cells (57), and inhibition of lipoxygenase inhibits proliferation (58) and stimulates apoptosis (59-61) of colon cancer cells. Similarly, blockage of the CysLT1 receptor enhances apoptosis of prostate cancer cells (62). Lipoxygenase further confers survival of neuroblastoma cells (63).

In erythrocytes, the increase in phosphatidylserine exposure is the result of Ca^{2+}-sensitive scrambling of the cell membrane (41, 42), the cell shrinkage due to activation of Ca^{2+}-sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, and exit of Cl^- together with osmotically obliged water, thus eventually leading to cell shrinkage (40). The cell shrinkage then contributes to the triggering of scrambling of the cell membrane (64). In addition to cell membrane scrambling and cell shrinkage, increased cytosolic Ca^{2+} activity affects the architecture of the cytoskeleton (65, 66) and activates several enzymes such as transglutaminase (67), phospholipases (68), calpain (67), protein kinases, and phosphatases (69, 70). Calpain-dependent degradation of membrane proteins leads to membrane blebbing, a further hallmark of eryptosis (41, 42).

Eryptosis eventually results in disposal of affected erythrocytes (2), as phosphatidylserine-exposing erythrocytes are engulfed by macrophages equipped with phosphatidylserine receptors (71) and thus eliminated from circulating blood (72). Phosphatidylserine-exposing erythrocytes could further adhere to the vascular wall and thus compromise microcirculation (73, 74). Suicidal erythrocytes have thus been proposed to participate in vascular injury of metabolic syndrome (75). Leukotrienes are known to interfere with microcirculation, an effect mainly attributed to their effect on vascular smooth muscle cells (76, 77). Along those lines, inhibition of leukotriene formation may counteract atherosclerosis (78, 79).

Leukotrienes may not only be involved in the triggering of eryptosis by energy depletion. Phosphatidylserine exposure in erythrocytes could be elicited by ligation of several surface antigens, such as glycophorin-C (80), the thrombospondin-1 receptor CD47 (81), and the death receptor CD95/Fas (82). Moreover, phosphatidylserine exposure or eryptosis is triggered by a wide variety of chemicals and drugs (83-87). Cell membrane scrambling is further stimulated by sepsis (88), iron deficiency (72), phosphate depletion (89), Hemolytic Uremic Syndrome (90), malaria (91, 92), Wilson's disease (93), glucose-phosphate dehydrogenase deficiency (94) and hemoglobinopathies (95). Future studies shall reveal, to which extent leukotrienes participate in the respective signalling of eryptosis.

Leukotrienes may similarly affect survival of nucleated cells. Apoptotic cells may release leukotrienes (96). Leukotriene D4 has been shown to induce apoptosis, an effect thought to be mediated by CysLT2 (97). Overexpression of CysLT1 rather attenuated apoptosis of PC12 cells (97), and CysLT1 inhibition enhanced apoptosis of intestinal cells (98). On the other hand, the CysLT1 inhibitor montelukast reversed leukocyte apotosis in chronic renal failure (99). The antiapoptotic effect of leukotriene D4 has been attributed to gene transcription (100), which cannot apply in erythrocytes. In nucleated cells, Ca^{2+} signaling may indeed play a dual role. While Ca^{2+} oscillations stimulate cell proliferation and confer cell survival (101), sustained increases in cytosolic Ca^{2+} could trigger suicidal cell death (102). The stimulation of Ca^{2+} entry in erythrocytes has uniformly been shown to stimulate suicidal erythrocyte death (2).

In conclusion, erythrocytes express the leukotriene receptor CysLT1. Energy depletion of erythrocytes triggers the formation of leukotrienes, which in turn stimulates caspases and activates cation channels, leading to Ca^{2+} entry with subsequent cell membrane scrambling and cell shrinkage. The present observations unravel a novel element in the complex machinery regulating suicidal erythrocyte death.

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