Original Paper

Sulindac Sulfide – Induced Stimulation of Eryptosis

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Key Words

Phosphatidylserine • Sulindac sulfide • Calcium • Cell volume • Ceramide • Eryptosis

Abstract

Background: Sulindac sulfide, a non-steroidal anti-inflammatory drug (NSAID), stimulates apoptosis of tumor cells and is thus effective against malignancy. In analogy to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, an apoptosis-like suicidal erythrocyte death, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine-exposure at the cell surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$) and ceramide formation. The present study explored, whether sulindac sulfide stimulates eryptosis. **Methods:** $[\text{Ca}^{2+}]_i$ was estimated from Fluo-3 fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from binding of fluorescent annexin-V, hemolysis from hemoglobin release, and ceramide abundance utilizing fluorescent antibodies. **Results:** A 48 h exposure to sulindac sulfide ($\leq 20 \mu\text{M}$) was followed by significant increase of $[\text{Ca}^{2+}]_i$, enhanced ceramide abundance, decreased forward scatter and increased percentage of annexin-V-binding erythrocytes. Sulindac sulfide triggered slight but significant hemolysis. Removal of extracellular Ca^{2+} significantly blunted, but did not abrogate the effect of sulindac sulfide ($20 \mu\text{M}$) on annexin-V-binding. **Conclusion:** Sulindac sulfide stimulates the suicidal death of erythrocytes or eryptosis, an effect paralleled by Ca^{2+} -entry, ceramide formation, cell shrinkage and phosphatidylserine-exposure.

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Introduction

Sulindac, a non-steroidal anti-inflammatory drug (NSAID) is used for the treatment of pain and a wide variety of inflammatory disorders [1]. Moreover, sulindac has been shown to counteract malignancy [2-9], an effect attributed at least in part to stimulation of apoptosis [9-15]. Mechanisms considered to participate in stimulation of tumor cell apoptosis by sulindac include altered regulation of gene expression [9, 16-19]. On the other hand, sulindac may induce apoptosis of tumor cells by increasing cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [13].

An increase of $[\text{Ca}^{2+}]_i$ is similarly able to trigger eryptosis, the suicidal death of erythrocytes, which is characterized by cell membrane scrambling and cell shrinkage [20]. In erythrocytes $[\text{Ca}^{2+}]_i$ may be increased by Ca^{2+} entry through Ca^{2+} -permeable cation channels [21, 22]. Cytosolic Ca^{2+} concentration activates Ca^{2+} -sensitive K^+ channels [23] leading to K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl loss together with osmotically obliged water, which results in cell shrinkage [24]. Cytosolic Ca^{2+} further triggers cell membrane scrambling with phosphatidylserine exposure at the cell surface [25]. The Ca^{2+} sensitivity of erythrocyte cell membrane scrambling is enhanced by ceramide, which similarly stimulates eryptosis [26]. Eryptosis is further triggered by energy depletion [27] and activation of caspases [28-32]. Eryptosis is modified by AMP activated kinase AMPK [22], cGMP-dependent protein kinase [33] and Janus-activated kinase JAK3 [34].

Phosphatidylserine exposed at the erythrocyte surface binds to endothelial CXCL16/SR-PSO leading to adherence of eryptotic cells to endothelial cells [35]. The adhesion of phosphatidylserine exposing erythrocytes to the vascular wall interferes with microcirculation [35-40]. Phosphatidylserine exposing erythrocytes further foster blood clotting [36, 41, 42]. Accordingly, excessive eryptosis may lead to thrombosis. Moreover, the rapid clearance of erythrocytes from circulating blood may lead to anemia [20]. Accordingly, substances triggering eryptosis could, at least in theory, trigger thrombosis on the one hand, and anemia on the other. Sulindac has indeed been reported to trigger anemia [43]. Whether or not the anemia was secondary to suicidal erythrocyte death, has, however, remained elusive.

The present study thus explored, whether sulindac sulfide increases erythrocyte $[\text{Ca}^{2+}]_i$ and elicits eryptosis.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes 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 (184/2003V). Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% (4 μL) in 1 ml of 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 48 h. Where indicated, erythrocytes were exposed to sulindac sulfide (Enzo, Lörrach, Germany), dissolved in DMSO solution at the indicated concentrations. In Ca^{2+} -free Ringer solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 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 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

To determine intracellular Ca^{2+} , erythrocytes were incubated *in vitro* at a hematocrit of 0.4% (4 μL) in 1 mL of 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 48 h. After incubation erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 30 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 at an excitation wavelength of 488 nm and emission wavelength of 530 nm by FACS analysis.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without sulindac sulfide, cells were stained for 1 h at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis in FL-1.

Ceramide formation was further determined by confocal microscopy. To this end, 20 μl aliquots of anti-ceramide antibody treated samples with or without sulindac pretreatment were smeared onto a glass slide, covered with a coverslip and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Measurement of hemolysis

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, 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 $\pm$ SD. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test or *t* test as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

Cytosolic Ca^{2+} concentration in erythrocytes was estimated from Fluo-3 fluorescence. To this end erythrocytes were loaded with Fluo-3/AM and the Fluo-3 fluorescence determined utilizing flow cytometry (FACS). Exposure of human erythrocytes to sulindac sulfide was followed by an increase of Fluo-3 fluorescence, an effect reaching statistical significance at 5 μM sulindac sulfide concentration (Fig. 1). Accordingly, sulindac sulfide increased cytosolic Ca^{2+} concentration.

Enhanced cytosolic Ca^{2+} concentrations are known to activate Ca^{2+} -sensitive K^+ channels resulting in KCl exit with osmotically obliged water, an effect leading to cell shrinkage. Thus, cell volume was estimated from forward scatter in FACS analysis. As illustrated in Fig. 2, the exposure of human erythrocytes to sulindac sulfide was followed by a decrease of forward scatter, an effect reaching statistical significance at 20 μM sulindac sulfide concentration.

Increased cytosolic Ca^{2+} concentration is further expected to trigger cell membrane scrambling resulting in phosphatidylserine exposure at the cell surface. Thus, additional experiments were performed to identify phosphatidylserine exposing erythrocytes with fluorescent annexin-V. The percentage of annexin-V-binding erythrocytes was determined by FACS analysis. As shown in Fig. 3, the percentage of annexin-V-binding erythrocytes increased following a 48 h exposure to sulindac sulfide. The effect reached statistical significance at 20 μM sulindac sulfide concentration.

Fig. 1. Effect of sulindac sulfide on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 20 μM sulindac sulfide. B. Arithmetic means $\pm$ SD ($n = 13$) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo-3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) sulindac sulfide (5-20 μM). *** ($p < 0.001$) indicates significant difference from the absence of sulindac sulfide (ANOVA).

Fig. 2. Effect of sulindac sulfide on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 20 μM sulindac sulfide. B. Arithmetic means $\pm$ SD ($n = 13$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) sulindac sulfide (5-20 μM). *** ($p < 0.001$) indicates significant difference from the absence of sulindac sulfide (ANOVA).

In an additional series of experiments the effect of sulindac sulfide on hemolysis was estimated from hemoglobin concentration in the supernatant. As illustrated in Fig. 3, exposure of erythrocytes for 48 h to sulindac sulfide was indeed followed by an increase of the extracellular hemoglobin concentration, an effect reaching statistical significance at 10 μM (Fig. 3 B). The percentage of hemolysed erythrocytes remained, however, one order of magnitude smaller than the percentage of phosphatidylserine exposing cells.

Further experiments aimed to gain some insight into the mechanisms underlying sulindac sulfide induced cell membrane scrambling. A first series of experiments addressed,

Fig. 3. Effect of sulindac sulfide on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 20 μ M sulindac sulfide. B. Arithmetic means $\pm$ SD of erythrocyte annexin-V-binding ($n = 13$) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of sulindac sulfide (5–20 μ M). For comparison, arithmetic means $\pm$ SD ($n = 4$) of the percentage of hemolysis is shown as grey bars. ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of sulindac sulfide (ANOVA).

Fig. 4. Effect of Ca^{2+} withdrawal on sulindac sulfide-induced annexin-V-binding. Arithmetic means $\pm$ SD ($n = 4$) of the percentage of annexin V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 20 μ M sulindac sulfide in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of calcium. *** ($p < 0.001$) indicates significant difference from the absence of sulindac sulfide (ANOVA) ### ($p < 0.001$) indicates significant difference from the respective values in the presence of Ca^{2+} .

whether the sulindac sulfide induced cell membrane scrambling required Ca^{2+} entry from the extracellular space. To this end, erythrocytes were exposed to 20 μ M sulindac sulfide either in the presence or in the nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 4, the effect of sulindac sulfide on the percentage of annexin-V-binding erythrocytes was significantly blunted in the nominal absence of Ca^{2+} . However, even in the nominal absence of Ca^{2+} , the percentage of annexin-V-binding erythrocytes was significantly higher in the presence than in the nominal absence of extracellular Ca^{2+} . Thus, stimulation of erythrocyte membrane scrambling by sulindac sulfide was in large part but not fully dependent on Ca^{2+} entry.

As Ca^{2+} sensitivity of cell membrane scrambling is known to be enhanced by ceramide, additional experiments were performed analysing the effect of sulindac sulfide treatment on ceramide formation. To this end, ceramide abundance at the erythrocyte surface was determined utilizing FITC-labeled anti-ceramide antibodies. As a result, a 48 h incubation

Fig. 5. Effect of sulindac sulfide on ceramide formation. A. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of sulindac sulfide (20 μ M). B. Arithmetic means $\pm$ SD ($n = 4$) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) sulindac sulfide (20 μ M). * ($p < 0.05$) indicates significant difference from control (absence of sulindac sulfide) (unpaired t test). C. Confocal microscopy of FITC-dependent fluorescence of human erythrocytes stained with anti-ceramide antibody following 48 h incubation in Ringer solution without (left) and with (right) 20 μ M sulindac sulfide.

in sulindac sulfide (20 μ M) significantly increased the ceramide-dependent fluorescence intensity (Fig. 5 B). The increase of ceramide abundance was further apparent from confocal microscopy (Fig. 5 C).

Discussion

The present study demonstrates that sulindac sulfide increases cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and triggers eryptosis, the suicidal death of erythrocytes, which is characterized by erythrocyte membrane scrambling and erythrocyte shrinkage. The concentration required for the effect on cell membrane scrambling is in the range of the *in vivo* plasma concentrations, which may approach some 10 μ M [44]. The free plasma concentration may be lower due to binding of the substance to plasma protein [45].

Sulindac has previously been shown to increase $[\text{Ca}^{2+}]_i$ in tumor cells [13]. In erythrocytes, the Ca^{2+} entry is secondary to activation of non-selective cation channels. The molecular identity of the Ca^{2+} permeable erythrocyte cation channels has remained enigmatic but apparently does involve the Ca^{2+} permeable cation channel TRPC6 [21]. The cation channels are sensitive to oxidative stress [46]. The stimulation of Ca^{2+} entry into erythrocytes parallels a similar phenomenon in tumor cells. In those cells, sulindac induced apoptosis involves, however,

a complex machinery eventually affecting gene expression [9, 16-19]. As erythrocytes lack nuclei, altered transcription is obviously not required for sulindac-induced suicidal cell death.

As shown earlier [23, 47], the increase of cytosolic Ca^{2+} activity opens Ca^{2+} -sensitive K^+ channels resulting in subsequent exit of K^+ following its chemical gradient, cell membrane hyperpolarization and potential driven Cl^- exit [20]. The cellular loss of KCl is accompanied by exit of osmotically obliged water [24] leading to cell shrinkage. Accordingly, sulindac decreases erythrocyte forward scatter. The effect on cell volume is small. Given the high sensitivity of a variety of cellular functions to cell volume, however, this small effect could well be relevant [48].

The increase of cytosolic Ca^{2+} activity further elicits cell membrane scrambling, which results in phosphatidylserine exposure at the erythrocyte surface [25, 49, 50]. The sulindac-induced cell membrane scrambling is significantly blunted in the nominal absence of extracellular Ca^{2+} , indicating that Ca^{2+} entry is indeed the major mechanism accounting for sulindac-induced suicidal erythrocyte death. However, removal of extracellular Ca^{2+} did not fully abrogate the scrambling effect of sulindac sulfide, indicating that sulindac sulfide-induced eryptosis involves additional mechanisms.

One of those mechanisms may be ceramide formation. Sulindac sulfide did increase the formation of ceramide, which sensitizes the erythrocytes for the scrambling effect of increased $[\text{Ca}^{2+}]_i$ [26, 51]. Ceramide similarly triggers apoptosis of nucleated cells [52]. Ceramide formation participates in the pathophysiology of several clinical disorders, such as lung inflammation, fibrosis and infection [53], cystic fibrosis [54], cardiovascular disease [55, 56], Wilson's disease [57], multiple sclerosis [58], major depression [52], Parkinson's disease [59], Alzheimer's disease [52, 60, 61] and diabetes [62-64].

It should be pointed out that even at higher concentrations, sulindac triggers eryptosis only in a subset of erythrocytes. The effect of sulindac on cytosolic Ca^{2+} , forward scatter, phosphatidylserine exposure and ceramide formation, is not homogeneous. Thus, the erythrocytes are not uniformly sensitive to the drug. The sensitivity of erythrocytes to triggers of eryptosis is influenced by erythrocyte age [65] and presumably less well defined properties of individual erythrocytes.

Similar to sulindac sulfide a wide variety of xenobiotics trigger eryptosis [28, 34, 66-75]. Enhanced eryptosis further contributes to the pathophysiology of several clinical disorders [20], including diabetes [32, 76, 77], renal insufficiency [78], hemolytic uremic syndrome [79], sepsis [80], sickle cell disease [81], malaria [57, 82-85], Wilson's disease [57], iron deficiency [86], phosphate depletion [87] and presumably metabolic syndrome [88].

In conclusion, sulindac sulfide elicits Ca^{2+} entry and ceramide formation, effects eventually leading to stimulation of cell membrane scrambling and cell shrinkage. Accordingly, similar to its proapoptotic effect on nucleated cells, sulindac sulfide stimulates eryptosis, the suicidal death of erythrocytes.

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