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Original Paper

Induction of Suicidal Erythrocyte Death by Novobiocin

Adrian Lupescu Rosi Bissinger Tabea Herrmann Gergely Oswald Kashif Jilani Florian Lang

Department of Physiology, Eberhard-Karls-University of Tuebingen, Tuebingen, Germany

Key Words
Phosphatidylserine • Calcium • Cell volume • Eryptosis

Abstract
Background: Novobiocin, an aminocoumarin antibiotic, interferes with heat shock protein 90 and hypoxia inducible factor dependent gene expression and thus compromises cell survival. Similar to survival of nucleated cells, erythrocyte survival could be disrupted by eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by phospholipd scrambling of the cell membrane with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$). The Ca^{2+} sensitivity of phospholipid scrambling is enhanced by ceramide. The present study explored, whether novobiocin elicits eryptosis. ***Methods:*** $[Ca^{2+}]_i$ was estimated from Fluo3-fluorescence, ceramide abundance utilizing fluorescent antibodies, cell volume from forward scatter, phosphatidylserine-exposure from annexin V binding. ***Results:*** A 48 hours exposure to novobiocin (500 μM) was followed by a significant increase of $[Ca^{2+}]_i$, decrease of forward scatter, increase of annexin-V-binding and enhanced ceramide formation. Removal of extracellular Ca^{2+} virtually abrogated the increase of annexin-V-binding following novobiocin exposure. ***Conclusions:*** Novobiocin stimulates eryptosis, an effect at least in part due to entry of extracellular Ca^{2+} and formation of ceramide.

Introduction

Novobiocin, an aminocoumarin antibiotic [1] produced by the actinomycete *Streptomyces niveus* [2], has antimicrobial activity with acceptable toxicity and bioavailability [3, 4]. The substance inhibits heat shock protein HSP90 [5], which is in turn important for tumorigenesis

Prof. Dr. Florian Lang

Physiologisches Institut, der Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618, E-Mail florian.lang@uni-tuebingen.de

and cancer cell survival [3, 4, 6, 7]. Moreover, novobiocin has been shown to interfere with the function of the transcription factor Hypoxia-inducible factor 1α (HIF1α) and thus with HIF1α-controlled gene expression [1]. Thus, novobiocin and its analogues compromize the survival of tumor cells and are considered for the treatment of malignancy [1, 3-5].

Similar to survival nucleated cells, survival of erythrocytes may be limited by suicidal erythrocyte death or eryptosis [8]. Hallmarks of eryptosis include erythrocyte shrinkage [9] and breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane with translocation of phosphatidylserine to the erythrocyte surface [8]. Triggers of eryptosis include increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) following Ca^{2+} entry through Ca^{2+}-permeable cation channels [10, 11] or a permeabilized erythrocyte cell membrane [12]. Increased $[Ca^{2+}]_i$ activates Ca^{2+}-sensitive K^+ channels [13] leading to K^+ exit, hyperpolarization and Cl^- exit thus resulting in cell shrinkage by cellular loss of KCl and osmotically obliged water [9]. Increased $[Ca^{2+}]_i$ further triggers cell membrane scrambling with translocation of phosphatidylserine from the inner leaflet of the cell membrane to the erythrocyte surface [14]. The cell membrane is sensitized to cytosolic Ca^{2+} by ceramide [15]. Further triggers of eryptosis include energy depletion [16], caspase activation [17-21] or deranged activity of AMP activated kinase AMPK [11], cGMP-dependent protein kinase [22], Janus-activated kinase JAK3 [23], casein kinase [24, 25], p38 kinase [26], PAK2 kinase [27] as well as sorafenib [28] and sunifinib [29] sensitive kinases.

Eryptosis could be elicited by a wide variety of xenobiotics [29-60] and excessive eryptosis is observed in several clinical disorders [8], such as diabetes [21, 61, 62], renal insufficiency [63], hemolytic uremic syndrome [64], sepsis [65], malaria [66-70], sickle cell disease [71], Wilson's disease [69], iron deficiency [72], malignancy [73], phosphate depletion [74], and metabolic syndrome [56].

The present study explored, whether novobiocin is able to induce eryptosis. To this end, $[Ca^{2+}]_i$, ceramide abundance, cell volume and phosphatidylserine abundance at the erythrocyte surface have been determined in human erythrocytes prior to and following exposure to novobiocin.

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% 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 48 h. Where indicated, erythrocytes were exposed to novobiocin (Enzo, Lörrach, Germany) 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 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+}

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 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

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.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody based assay was used. After incubation with and without novobiocin, cells were stained for 1 h at 37°C with 1 µg/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 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis in FL-1.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored, whether novobiocin influences erythrocyte survival by triggering eryptosis, the suicidal death of erythrocytes. The major hallmark of eryptosis is cell membrane scrambling with translocation of phosphatidylserine in the cell membrane from inside to the outer surface. Phosphatidylserine exposing erythrocytes were identified from binding of fluorescent FITC-annexin-V, which was determined by flow cytometry. As illustrated in Fig. 1, a 48 hours exposure of human erythrocytes from healthy individuals to novobiocin was followed by an increase of the percentage annexin-V-binding erythrocytes, an effect reaching statistical significance at 500 µM novobiocin concentration (Fig 1A,B).

In parallel experiments, the effect of novobiocin treatment on hemolysis was tested. To this end, the percentage of hemolysed erythrocytes was estimated from the hemoglobin concentration in the supernatant. As a result, a 48 hours exposure of erythrocytes to novobiocin resulted in an increase of the percentage hemolysed erythrocytes from 1.7 ± 0.1 % (n = 4) in the absence of novobiocin to 2.3 ± 0.1% (n = 4) at 100 µM novobiocin, 2.6 ± 0.2% (n = 4) at 300 µM novobiocin and 3.0 ± 0.2% (n = 4) at 500 µM novobiocin, respectively. The percentage of hemolytic erythrocytes thus remained clearly smaller than the percentage of annexin-V-binding erythrocytes.

A second hallmark of eryptosis is cell shrinkage. Thus, cell volume was estimated from forward scatter in flow cytometry. As shown in Fig. 2A,B, novobiocin treatment resulted in a decrease of forward scatter, an effect reaching statistical significance at 500 µM novobiocin concentration.

Cell membrane scrambling and cell shrinkage are both triggered by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Accordingly, $[Ca^{2+}]_i$ was determined utilizing Fluo3 fluorescence. To this end, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in flow cytometry following prior incubation of the erythrocytes in Ringer solution without

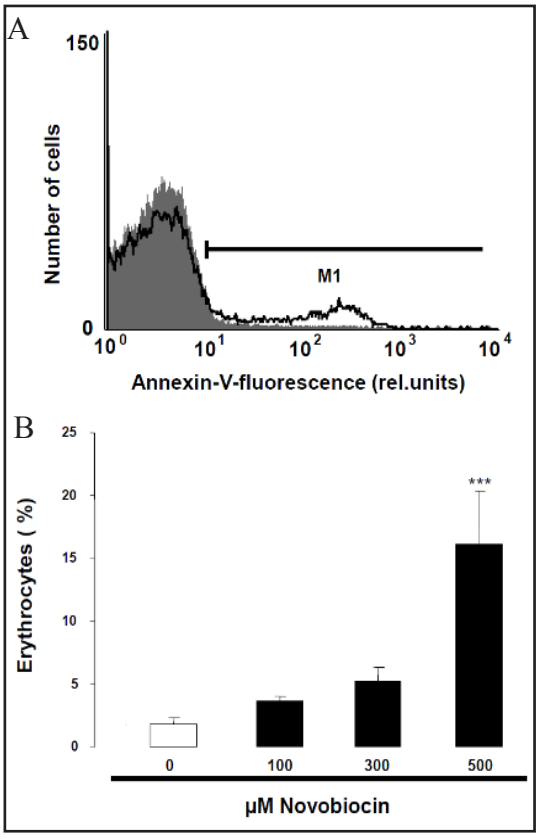

Fig. 1. Effect of novobiocin on phosphatidylserine exposure. A. Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 500 µM novobiocin. B. Arithmetic means ± SEM (n = 8) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of novobiocin (100-500 µM). *** (p<0.001) indicates significant differences from the absence of novobiocin (ANOVA).

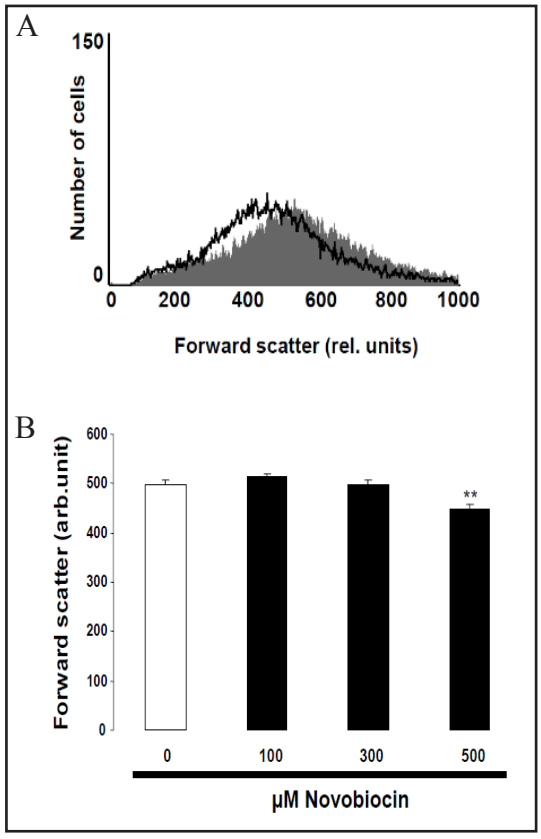

Fig. 2. Effect of novobiocin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 500 µM novobiocin. B. Arithmetic means ± SEM (n = 8) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) novobiocin (100-500 µM). ** (p<0.01) indicates significant difference from the absence of novobiocin (ANOVA).

or with novobiocin. As shown in Fig. 3A,B, exposure of human erythrocytes to novobiocin increased Fluo3 fluorescence, an effect reaching statistical significance at 500 µM novobiocin concentration.

A further series of experients explored, whether extracellular Ca^{2+} entry was required for the effect of novobiocin on cell membrane scrambling. To this end, erythrocytes were exposed to 500 µM novobiocin for 48 hours either in the presence of 1 mM Ca^{2+} or in the absence of Ca^{2+} and the presence of Ca^{2+} chelator EGTA (1 mM). As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of novobiocin on annexin-V-binding. As a matter of fact, in the absence of extracellular Ca^{2+} novobiocin had no significant effect on annexin V binding.

The sensitivity of cell membrane scrambling to cytosolic Ca^{2+} activity could be enhanced by ceramide. An additional series of experiments was thus performed in order to explore the effect of novobiocin treatment on ceramide formation. Ceramide abundance was determined utilizing FITC-labeled anti-ceramide antibodies. As shown in Fig. 5A,B, novobiocin significantly enhanced the ceramide-dependent fluorescence.

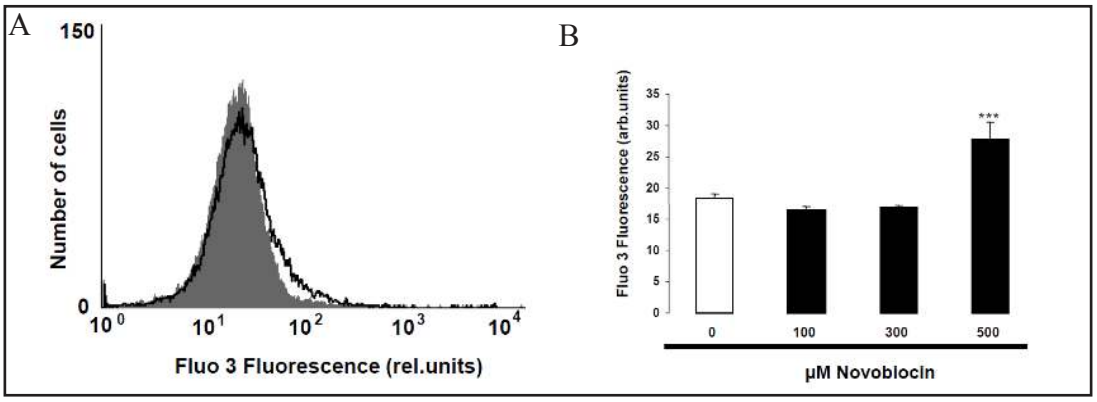

Fig. 3. Effect of novobiocin on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 500 µM novobiocin. B. Arithmetic means ± SEM (n = 8) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) novobiocin (100-500 µM). *** ($p<0.001$) indicates significant difference from the absence of novobiocin (ANOVA).

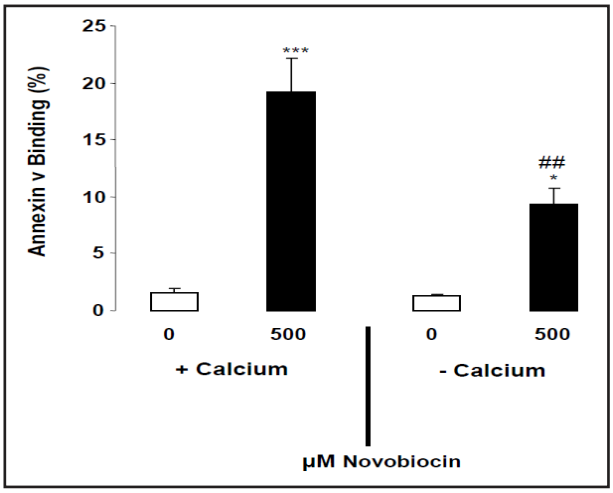

Fig. 4. Effect of Ca^{2+} withdrawal on novobiocin-induced annexin-V-binding. Arithmetic means ± SEM (n =5) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 500 µM novobiocin in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $- Ca^{2+}$) of calcium. * ($p<0.05$), *** ($p<0.001$), indicate significant difference from the respective values in absence of novobiocin, ## ($p<0.01$) indicates significant difference from the respective value in the presence of Ca^{2+} (ANOVA).

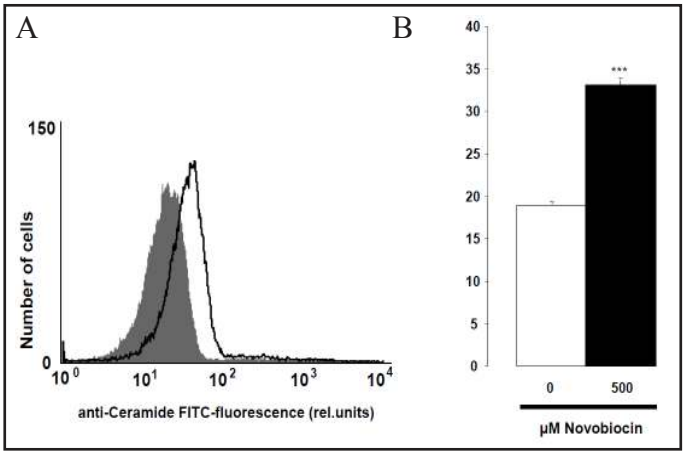

Fig. 5. Effect of novobiocin on ceramide formation. A. Original histogram of ceramide-dependent fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 500 µM novobiocin. B. Arithmetic means ± SEM (n = 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bar) novobiocin (500 µM). *** ($p<0.001$) indicates significant difference from control (absence of novobiocin) (ANOVA).

Discussion

The present study reveals a novel effect of novobiocin, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Treatment of human erythrocytes with novobiocin is followed

by decrease of cell volume and translocation of phosphatidylserine from the inner leaflet of the cell membrane to the outer cell membrane surface. The concentrations required to trigger eryptosis were within the range of concentrations (300-500 μM) reported in humans following oral administration [75]. Those concentrations have been shown for instance to decrease viability of nucleated cells [76], to enhance NO accumulation [77] and to induce metazoan apoptosis [78]. Even higher concentrations of novobiocin have been employed to stimulate degradation of Hsp90-dependent client proteins [79-81], to inhibit self-splicing of primary transcripts of the phage T4 thymidylate synthase gene [82] and to inhibit intestinal secretion [83]. However, at lower concentrations, novobiocin may exert a variety of effects, including inhibition of the organic anion transporters hOAT1, hOAT3, and hOAT4 [84] and neurotoxicity [85]. The relevance of the present observations for *in vivo* conditions remains uncertain, as it cannot be excluded that the drug is bound to proteins *in vivo* and the free concentration is lower than that triggering eryptosis *in vitro*.

The effect of novobiocin on erythrocyte cell volume was in part secondary to the effect of novobiocin on Ca^{2+} entry from extracellular space with the resultant increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Ca^{2+} may enter through endogenous Ca^{2+} permeable non-selective cation channels somehow involving TRPC6 channels [10]. The mechanism involved in the activation of those channels remained elusive. It is noteworthy, however, that the Ca^{2+} permeable channels could be activated by oxidative stress [86].

The increase of $[Ca^{2+}]_i$ leads to activation of Ca^{2+} sensitive K^+ channels [13, 87] resulting in K^+ exit, cell membrane hyperpolarisation, Cl^- exit and cellular loss of KCl with osmotically obliged water [9]. Increase of $[Ca^{2+}]_i$ further triggers cell membrane scrambling.

Besides stimulating Ca^{2+} entry, novobiocin triggers the formation of ceramide, which is known to enhance the sensitivity of the scrambling machinery to $[Ca^{2+}]_i$ [15]. Ceramide is produced by a sphingomyelinase, which is activated by platelet activating factor [15]. How novobiocin leads to increase of ceramide abundance remained, however, again elusive.

Eryptosis is a physiological mechanism eliminating defective erythrocytes [60]. Eryptosis may precede hemolysis thus preventing release of cellular hemoglobin, which would otherwise be filtered in renal glomerula and subsequently occlude renal tubules [88]. The activation of K^+ channels serves to counteract cell swelling, which would eventually lead to rupture of the cell membrane and subsequent hemolysis. Eryptosis may be particularly important for the elimination of infected erythrocytes. Eryptosis is stimulated following infection of erythrocytes with *Plasmodium falciparum*, a pathogen opening the cation channels by inducing oxidative stress [67]. The eryptosis and subsequent clearance of eryptotic erythrocytes leads to removal of infected erythrocytes and thus counteracts parasitemia [67]. Along those lines novobiocin and/or other substances triggering eryptosis may, at least in theory, accelerate eryptosis of infected erythrocytes and thus lead to a more favourable clinical course of the disease [67].

Excessive eryptosis may, however, be pathophysiologically relevant [60]. Eryptotic erythrocytes are rapidly cleared from circulating blood and thus, triggering of eryptosis may lead to anemia [8]. Phosphatidylserine exposing erythrocytes further bind to endothelial CXCL16/SR-PSO [89]. The adherance of phosphatidylserine exposing erythrocytes to the vascular wall could interfere with blood flow [89-94]. Moreover, phosphatidylserine exposing erythrocytes may trigger blood clotting and thrombosis [90, 95, 96]. Thus, novobiocin and/or other substances triggering eryptosis may, at least in theory, foster the development of anemia and thrombosis.

Conclusion

Novobiocin stimulates the suicidal death of erthrocytes or eryptosis, an effect at least in part due to stimulation of Ca^{2+} entry and triggering of ceramide formation.

Conflict of Interests

The authors state that there are no conflicts.

Acknowledgements

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