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Original Paper

Nocodazole Induced Suicidal Death of Human Erythrocytes

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background: The microtubule assembly inhibitor nocodazole has been shown to trigger caspase-independent mitotic death and caspase dependent apoptosis. Similar to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress and ceramide. The present study explored, whether and how nocodazole induces eryptosis. **Methods:** Flow cytometry was employed to determine phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, the abundance of reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein (DCF) diacetate dependent fluorescence as well as ceramide surface abundance utilizing specific antibodies. Tubulin abundance was quantified by TubulinTracker™ Green reagent and visualized by confocal microscopy. **Results:** A 48 hours exposure of human erythrocytes to nocodazole ($\geq 30 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells without significantly modifying average forward scatter. Nocodazole significantly increased Fluo3-fluorescence, significantly increased DCF fluorescence and significantly increased ceramide surface abundance. The effect of nocodazole on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+} and was not modified in the presence of Caspase 3 inhibitor zVAD (1 μM). Nocodazole treatment reduced the content of total tubulin. **Conclusions:** Nocodazole triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of Ca^{2+} entry, oxidative stress and ceramide.

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Introduction

Nocodazole interferes with microtubule assembly [1-14] thus interfering with mitosis due to formation of multipolar spindles [15, 16] and leading to cell cycle arrest in G2/M [8, 17-19]. Moreover, nocodazole causes cellular bulging and/or bending [20]. By interacting with microtubule function [21] nocodazole or related substances trigger caspase-independent mitotic death [22] or caspase dependent apoptosis [2, 5, 7, 10, 13, 16, 17, 23-28]. The triggering of apoptosis involves downregulation of mTOR [29], inactivation of the antiapoptotic Bcl-2 [2, 4, 23, 29] and mitochondrial depolarization [23]. Nocodazole is thus effective against malignancy [2, 5, 14, 15, 17, 21]. On the other hand, nocodazole counteracts TNF- α -induced activation of the mitogen-activated protein kinase p38 [30].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis [31], the suicidal death of erythrocytes characterized by cell shrinkage [32] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [31]. Triggers of eryptosis include increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i), ceramide [33], oxidative stress [31], energy depletion [31] activated caspases [34, 35], stimulated activity of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [31], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, mitogen- and stress-activated kinase MSK1/2 and sorafenib/sunitinib sensitive kinases [31, 36].

The present study explored whether nocodazole induces eryptosis. To this end, human erythrocytes from healthy volunteers were treated with nocodazole and phosphatidylserine surface abundance, cell volume, [Ca²⁺]_i, reactive oxygen species (ROS) and ceramide abundance determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples 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 (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21°C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl₂, at 37°C for 48 h. Where indicated, erythrocytes were exposed to nocodazole (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 μ l cell suspension was washed in Ringer solution containing 5 mM CaCl₂ 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. The annexin-V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Intracellular Ca²⁺

After treatment, erythrocytes were washed in Ringer solution and then loaded with 5 μ M Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution. The cells were incubated at 37°C for 30 min and washed once in Ringer solution. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μ l Ringer. Then, Ca²⁺-dependent 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 hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) and the supernatants were collected. 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.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in PBS containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed in PBS. The DCFDA-loaded erythrocytes were resuspended in 200 μ l Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 μ g/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. 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 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Tubulin staining

For studying the effect of nocodazole on tubulin in human erythrocytes TubulinTracker™ Green reagent (Oregon Green® 488 Taxol; bis-acetate, Thermo Fisher Scientific, MA, USA) was used. Briefly, treated samples were stained with TubulinTracker (250 nM) for 30 min in the dark at 37°C. The erythrocytes were washed twice and finally resuspended in 200 μ l Ringer solution containing 5 mM CaCl₂. For flow cytometry, TubulinTracker-dependent fluorescence of the samples was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD). For confocal microscopy, 60 μ l of each sample were spread onto a glass slide and dried for 15 minutes at RT. The slides were covered with PROlong Gold antifade reagent (Invitrogen, Darmstadt Germany). Images were taken on a Zeiss LSM 5 EXCITER confocal laser-scanning microscope or with phase light (Carl Zeiss MicroImaging, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics

Data are expressed as arithmetic means \pm 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 aimed to possibly disclose an effect of nocodazole on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. In order to identify phosphatidylserine exposing erythrocytes the erythrocytes were incubated with annexin-V which tightly binds to surface phosphatidylserine. Annexin-V abundance was quantified by flow cytometry. The measurements were made following a 48 hours incubation in Ringer solution without or with nocodazole (15 - 60 μ g/ml). As illustrated in Fig. 1, a 48 hours exposure to nocodazole increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 30 μ g/ml nocodazole.

In order to estimate erythrocyte volume, forward scatter was determined utilizing flow cytometry following a 48 hours incubation in Ringer solution without or with nocodazole

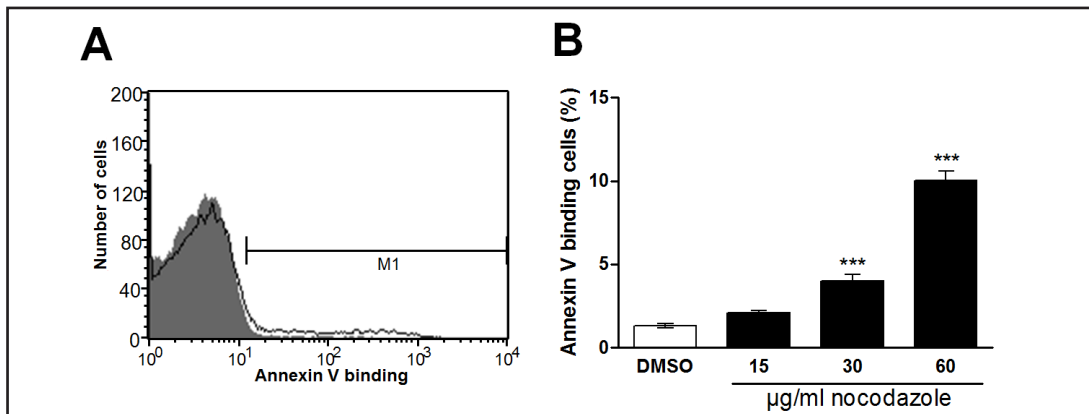


Fig. 1. Effect of nocodazole on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line). B. Arithmetic means ± SEM (n = 8) of the percentage of annexin-V-binding erythrocytes (black bars) following incubation for 48 hours to Ringer solution without or with presence of nocodazole (15 - 60 µg/ml). ***($p < 0.001$) indicates significant difference from the absence of nocodazole (ANOVA).

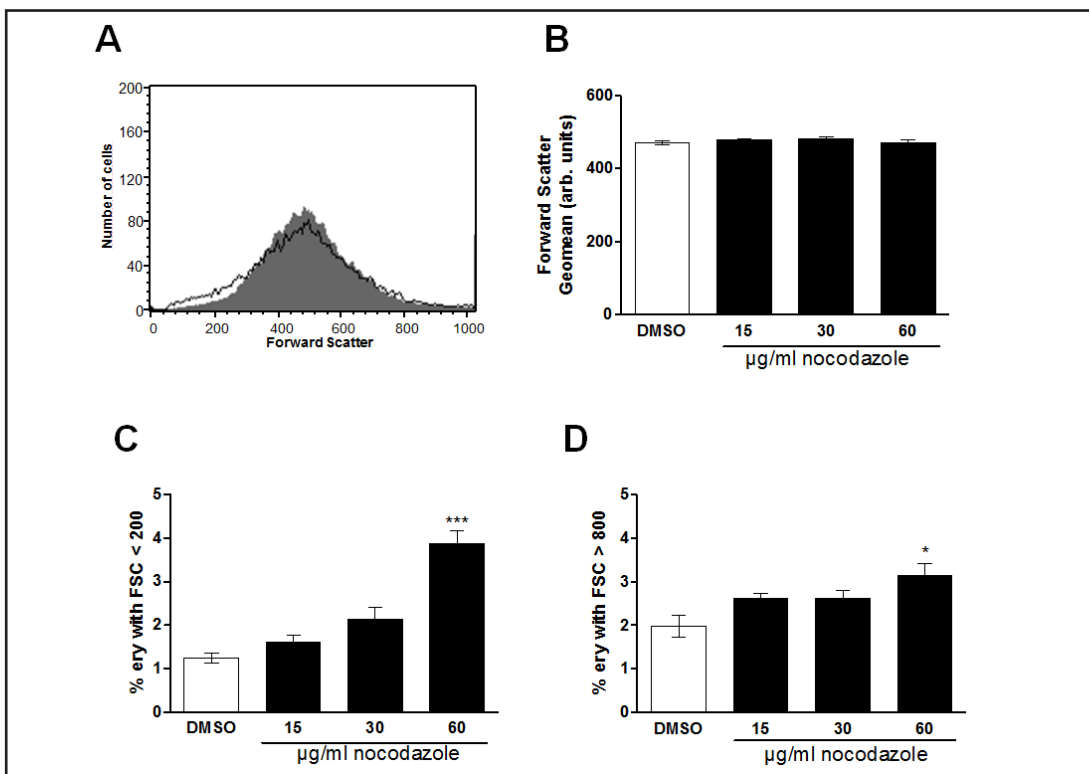


Fig. 2. Effect of nocodazole on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line). B. Arithmetic means ± SEM (n = 8) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (15 - 60 µg/ml). C. Arithmetic means ± SEM (n = 8) of the percentage erythrocytes with forward scatter (FSC) < 200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (15 - 60 µg/ml). D. Arithmetic means ± SEM (n = 8) of the percentage erythrocytes with forward scatter (FSC) > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (15 - 60 µg/ml). *($p < 0.05$), ***($p < 0.001$) indicate significant difference from the absence of nocodazole (ANOVA).

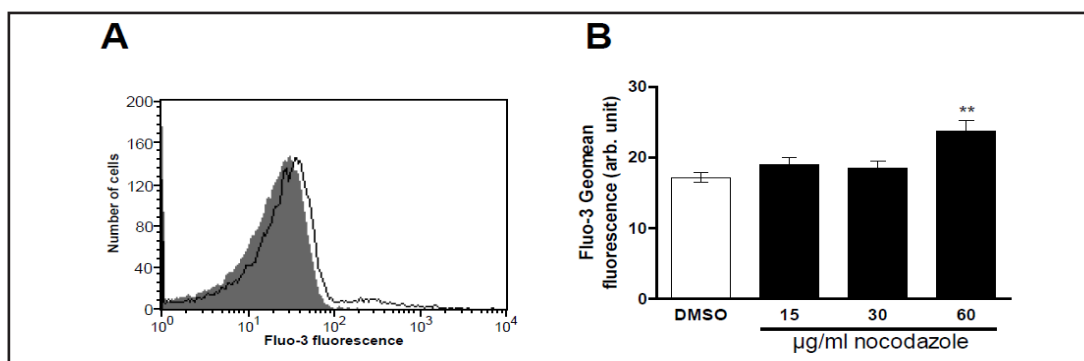


Fig. 3. Effect of nocodazole on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 $\mu\text{g/ml}$ nocodazole (black line). B. Arithmetic means \pm SEM ($n = 8$) of the Fluo3-fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (15 – 60 $\mu\text{g/ml}$). **($p < 0.01$) indicate significant difference from the absence of nocodazole (ANOVA).

(15 – 60 $\mu\text{g/ml}$). As shown in Fig. 2, at none of the applied concentrations, nocodazole had a significant effect on average erythrocyte forward scatter. Close inspection of Fig. 2A reveals, however, that exposure of erythrocytes to nocodazole was followed by an increase of the percentage of both small (Fig. 2C) and large (Fig. 2D) erythrocytes, alterations reaching significance at nocodazole concentrations of 60 $\mu\text{g/ml}$.

Hemolysis was estimated from the hemoglobin concentration in the supernatant which was determined by photometry. As a result, following a 48 hours incubation, the percentage of hemolytic erythrocytes was similar in the absence of nocodazole ($3.4 \pm 0.2\%$ $n = 12$) and in the presence of 15 $\mu\text{g/ml}$ ($4.4 \pm 0.2\%$ $n = 12$) or 30 $\mu\text{g/ml}$ ($4.7 \pm 0.2\%$ $n = 12$) nocodazole. Exposure to 60 $\mu\text{g/ml}$ nocodazole was, however, followed by a significant increase of hemolysis ($11.1 \pm 0.9\%$ $n = 12$).

Cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) was determined by Fluo3-fluorescence. As illustrated in Fig. 3, a 48 hours exposure to nocodazole increased the Fluo3-fluorescence, an effect reaching statistical significance at 60 $\mu\text{g/ml}$ nocodazole. In order to test whether the stimulation of cell membrane scrambling by nocodazole requires the entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 60 $\mu\text{g/ml}$ nocodazole in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of nocodazole on annexin-V-binding. Nevertheless, even in the absence extracellular Ca^{2+} nocodazole significantly increased the percentage of annexin-V-binding erythrocytes. Thus, nocodazole-induced cell membrane scrambling was in part but not fully due to entry of extracellular Ca^{2+} . Neither in the presence nor in the absence of extracellular Ca^{2+} did nocodazole significantly modify average forward scatter.

To test whether a 48 hours incubation with nocodazole modified the effect of excessive $[\text{Ca}^{2+}]_i$ on cell membrane scrambling or forward scatter, erythrocytes were exposed for 60 min to Ca^{2+} ionophore ionomycin (1 μM). As a result, ionomycin increased the percentage of annexin-V-binding erythrocytes to similarly high levels in erythrocytes incubated with nocodazole (from $4.8 \pm 0.3\%$ to $35.8 \pm 1.6\%$ $n = 4$) and in erythrocytes incubated without nocodazole (from $1.6 \pm 0.2\%$ to $38.7 \pm 1.8\%$ $n = 4$). Ionomycin decreased the forward scatter to similarly low levels in erythrocytes incubated with nocodazole (from $432 \pm 8.8\%$ to $124 \pm 1.9\%$, $n = 4$) and in erythrocytes incubated without nocodazole (from $440 \pm 8.8\%$ to $123 \pm 4.4\%$, $n = 4$).

Additional experiments were performed to elucidate the effect of nocodazole on oxidative stress. Reactive oxygen species (ROS) were estimated utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. As illustrated in Fig. 5, a 48 hours exposure to nocodazole increased the DCF fluorescence, an effect reaching statistical significance at 30 $\mu\text{g/ml}$ nocodazole.

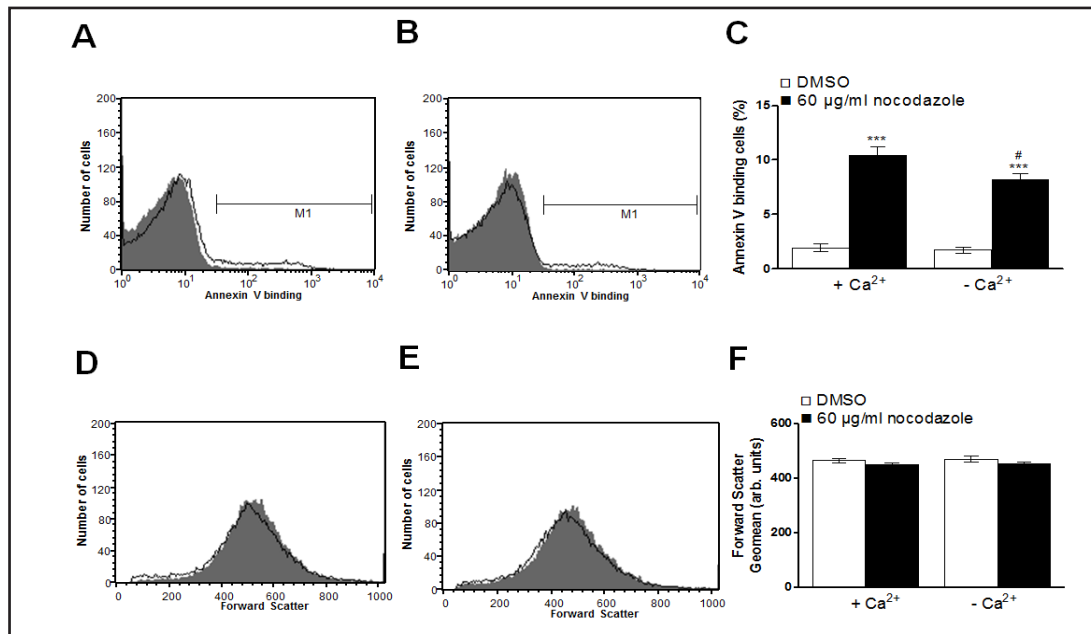


Fig. 4. Ca²⁺ sensitivity of nocodazole -induced phosphatidylserine exposure and erythrocyte shrinkage. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line) in the presence (A) and absence (B) of extracellular Ca²⁺. C. Arithmetic means ± SEM (n = 12) of the percentage of annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) nocodazole (60 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. D,E. Original histogram of erythrocyte forward scatter following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line) in the presence (D) and absence (E) of extracellular Ca²⁺. F. Arithmetic means ± SEM (n = 12) of erythrocyte forward scatter after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) nocodazole (60 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. ***(*P*<0.001) indicates significant difference from the absence of nocodazole, #(*p*<0.05) indicates significant difference from the presence of Ca²⁺ (ANOVA).

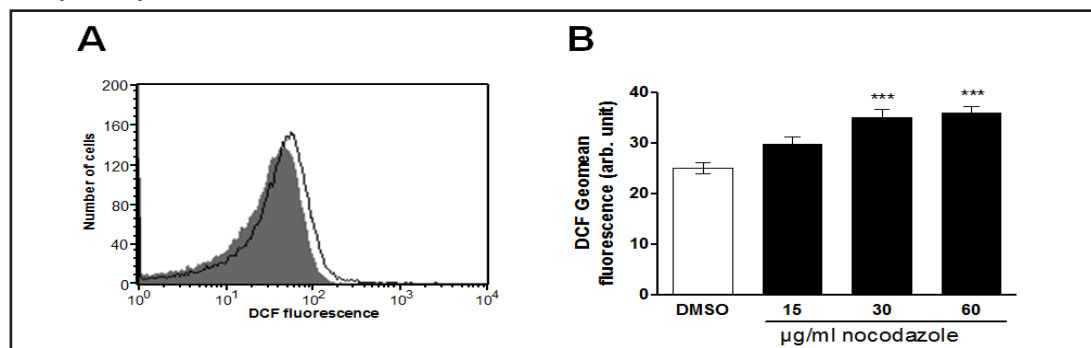


Fig. 5. Effect of nocodazole on erythrocyte ROS formation. A. Original histogram of DCF fluorescence in erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line). B. Arithmetic means ± SEM (n = 8) of the DCF fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (15 – 60 µg/ml). ***(*p*<0.001) indicates significant difference from the absence of nocodazole (ANOVA).

Eryptosis could further be stimulated by ceramide. Ceramide abundance at the erythrocyte surface was thus quantified with flow cytometry utilizing specific antibodies. As

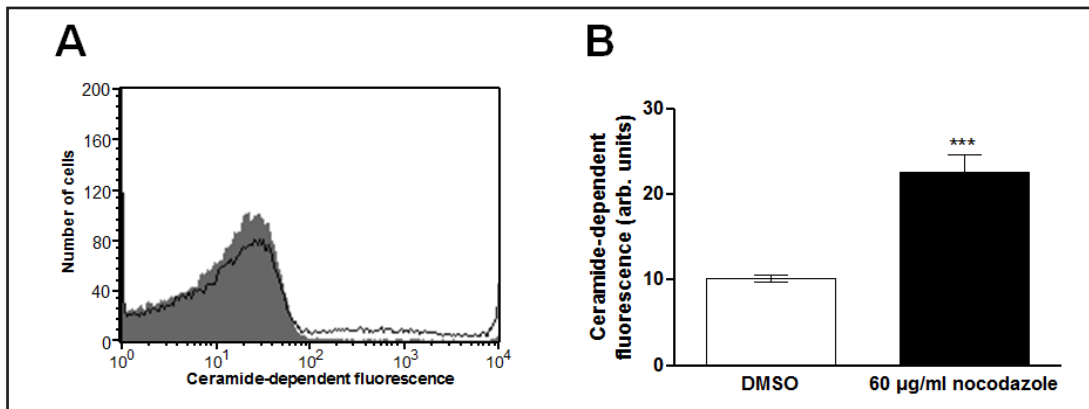


Fig. 6. Effect of nocodazole on ceramide abundance at the erythrocyte surface. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (grey area) and with presence of 60 µg/ml nocodazole (black line). B. Arithmetic means \pm SEM (n = 9) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (60 µg/ml). ***($p < 0.001$) indicates significant difference from the absence of nocodazole (Paired t test).

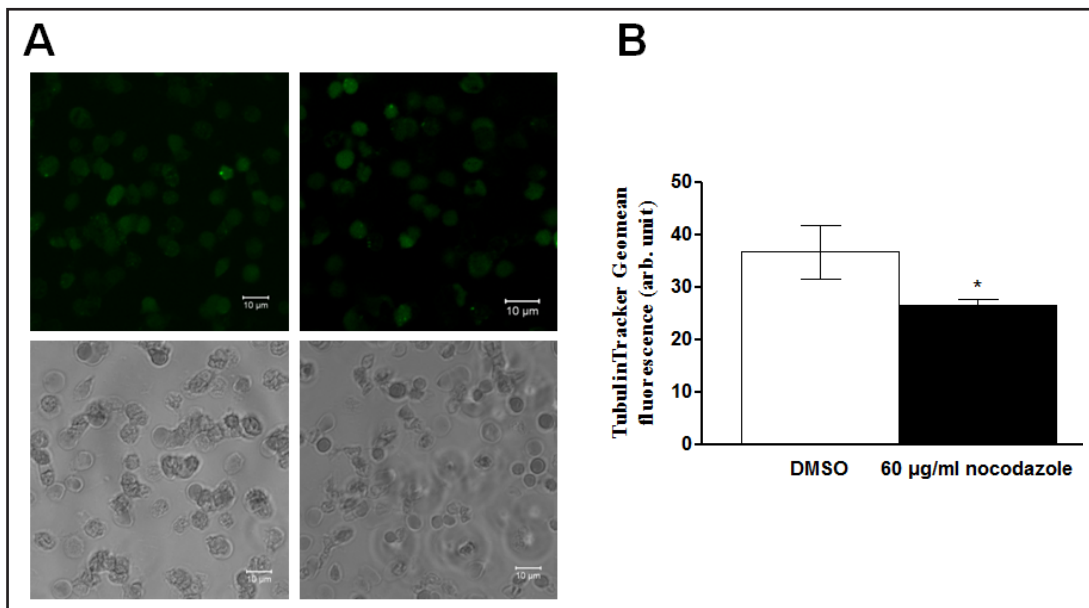


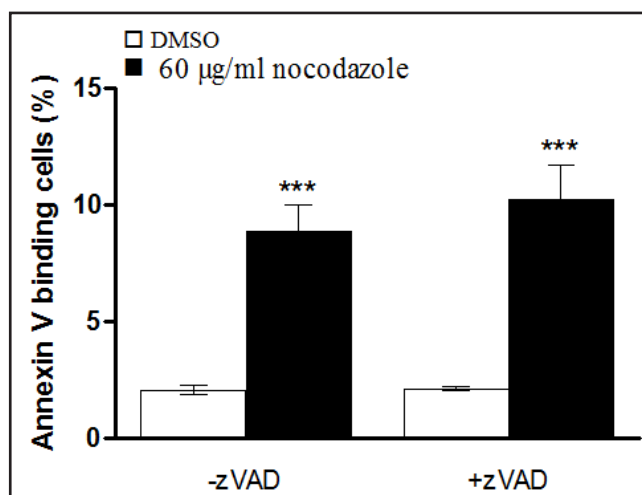
Fig. 7. Effect of nocodazole on tubulin abundance. A. Confocal images of tubulin abundance in erythrocytes following exposure for 48 hours to Ringer solution with solvent DMSO (left panel) and with presence of 60 µg/ml nocodazole (right panel). B. Arithmetic means \pm SEM (n = 4) of the TubulinTracker abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) nocodazole (60 µg/ml). *($p < 0.05$) indicates significant difference from the absence of nocodazole (Paired t test).

illustrated in Fig. 6, a 48 hours exposure to nocodazole (60 µg/ml) significantly increased the ceramide abundance.

To investigate the effect of nocodazole on tubulin in erythrocytes, TubulinTracker reagent was utilized in both flow cytometry and confocal microscopy. As illustrated in Fig. 7, 48 h treatment of erythrocytes with nocodazole (60 µg/ml) significantly reduced the TubulinTracker-dependent fluorescence.

To explore whether the stimulation of cell membrane scrambling by nocodazole required caspase activity, erythrocytes were exposed for 48 h to 60 µg/ml nocodazole either

Fig. 8. Insensitivity of nocodazole induced phosphatidylserine exposure to caspase inhibitor zVAD. Arithmetic means \pm SEM (n = 9) of the percentage of annexin-V-binding erythrocytes following incubation for 48 hours to Ringer solution without (white bars) or with (black bars) nocodazole (60 μ g/ml) treatment in the absence (-zVAD) or presence (+zVAD) of the pancaspase inhibitor zVAD (1 μ M). ***($p < 0.001$) indicates significant difference from the absence of nocodazole (ANOVA).



in the absence or presence of the pancaspase inhibitor zVAD (1 μ M). As illustrated in Fig. 8, zVAD did not significantly modify the effect of nocodazole on annexin-V-binding.

Discussion

The present observations demonstrate that nocodazole triggers cell membrane scrambling, the key event of suicidal erythrocyte death or eryptosis. The concentrations required for the toxic effect are higher than those required for disassembly of microtubules [13] but are similar to those previously employed to trigger cell cycle arrest [37].

The sensitivity to nocodazole may be particularly high in clinical conditions associated with enhanced eryptosis, such as dehydration [38], hyperphosphatemia [39] chronic kidney disease (CKD) [40-43], hemolytic-uremic syndrome [44], diabetes [45], hepatic failure [46], cardiac failure [47], malignancy [48], sepsis [49], fever [31, 50], sickle-cell disease [31], beta-thalassemia [31], Hb-C and G6PD-deficiency [31], Wilsons disease [51] as well as advanced age [52]. Moreover, the effect of nocodazole may be compounded by other eryptosis stimulating xenobiotics [38, 39, 41, 48, 53-106].

The stimulation by nocodazole of cell membrane scrambling was in part due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) and was thus significantly blunted by removal of extracellular Ca^{2+} . However, even in the absence of extracellular Ca^{2+} nocodazole treatment was followed by stimulation of cell membrane scrambling, pointing to additional mechanisms in the stimulation of eryptosis by nocodazole. Those mechanisms presumably include oxidative stress and ceramide.

Nocodazole did not significantly modify the average forward scatter but increased the percentage of both, shrunken and swollen erythrocytes. The cell shrinkage could be explained at least partially by increase of $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [32]. The mechanism driving nocodazole induced erythrocyte swelling remains elusive. A candidate mechanism could be impairment of Na^+/K^+ ATPase with subsequent cellular accumulation of Na^+ with Cl^- . Whatever mechanism is involved in nocodazole induced cell swelling, it leads to swelling of only a small subset of erythrocytes.

We observed a moderate decrease of tubulin abundance following nocodazole treatment. Whether this effect contributes to the stimulation of eryptosis, remains uncertain. In blood platelets Ca^{2+} induced apoptosis was seemingly insensitive to cytoskeletal rearrangement [107].

The nocodazole induced eryptosis was insensitive to the pancaspase inhibitor zVAD. Accordingly, the stimulation of cell membrane scrambling by nocodazole does apparently

not require caspases. Suicidal death of nucleated cells may similarly be independent from caspase activation [108-110]. Suicidal cell death may involve activation of other enzymes. Eryptosis, for instance, may involve activation of calpain [31].

Stimulation of eryptosis may lead to anemia due to rapid clearance of eryptotic erythrocytes from circulating blood [31]. Eryptosis further leads to adherence of phosphatidylserine exposing erythrocytes to the vascular wall [111], to stimulation of blood clotting and to triggering of thrombosis [112-114]. Accordingly, enhanced eryptosis may lead to impairment of microcirculation [33, 112, 115-118].

In conclusion, nocodazole triggers eryptosis with cell membrane scrambling, an effect paralleled by and in part due to increase of cytosolic Ca²⁺ activity, oxidative stress and ceramide.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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