Trifluoperazine-Induced Suicidal Erythrocyte Death and S-Nitrosylation Inhibition, Reversed by the Nitric Oxide Donor Sodium Nitroprusside

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Key Words
Phosphatidylserine • Eryptosis • Calcium • Nitric oxide • Trifluoperazine

Abstract
Background and Purpose: The high potency antipsychotic drug trifluoperazine (10-[3-(4-methyl-1-piperazinyl)-propyl]-2-(trifluoromethyl)-(10)H-phenothiazine dihydrochloride; TFP) may either counteract or promote suicidal cell death or apoptosis. Similar to apoptosis, erythrocytes may enter eryptosis, characterized by phosphatidylserine exposure at the cell surface and cell shrinkage. Eryptosis can be stimulated by an increase in cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]) and inhibited by nitric oxide (NO). We explored whether TFP treatment of erythrocytes induces phosphatidylserine exposure, cell shrinkage,
and calcium influx, whether it impairs S-nitrosylation and whether these effects are inhibited by NO. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, [Ca\(^{2+}\)] from Fluo3-fluorescence, and protein nitrosylation from fluorescence switch of the Bodipy-TMR / Sypro Ruby signal. **Results:** Exposure of human erythrocytes to TFP significantly enhanced the percentage of annexin-V-binding cells, raised [Ca\(^{2+}\)], and decreased S-nitrosylation. The effect of TFP on annexin-V-binding was not affected by removal of extracellular Ca\(^{2+}\) alone, but was significantly inhibited by pre-treatment with sodium nitroprusside (SNP), an effect significantly augmented by additional removal of extracellular Ca\(^{2+}\). A 3 hours treatment with 0.1 µM Ca\(^{2+}\) ionomycin triggered annexin-V-binding and cell shrinkage, effects fully reversed by removal of extracellular Ca\(^{2+}\). **Conclusions:** TFP induces eryptosis and decreases protein S-nitrosylation, effects blunted by nitroprusside. The effect of nitroprusside is attenuated in the presence of extracellular Ca\(^{2+}\).

**Introduction**

Trifluoperazine (10-[3-(4-methyl-1-piperazinyl)-propyl]-2-(trifluoromethyl)-(10) H-phenothiazine dihydrochloride; TFP) [1], a high potency antipsychotic drug used for the treatment of schizophrenia and schizophrenia-like conditions [2-4], has further been used as analgesic in patients with neuropathic pain due to sickle cell disease [5]. Side effects of TFP include movement disorders [2-5] and sedation [5]. TFP is effective in part by reorganization of the cytoskeletal architecture [6], binding to and inhibiting of Ca\(^{2+}/\)calmodulin [7, 8], which affects numerous proteins in the erythrocytes [9], including the plasma membrane Ca\(^{2+}\) pump [10] and eNOS [9]. Further interaction partners of TFP include protein kinase II\(\alpha\) [5, 11], Wnt/\(\beta\)-catenin signaling [12], and DNA-dependent protein kinase [13]. TFP may depolymerize actin filaments [14], decrease Bcl-2 and increase Bax protein abundance [14]. Moreover, TFP increases early growth response gene-1 (Egr-1) expression [15] and the phosphorylation of ERK and JNK [14]. TFP inhibits P-glycoprotein expression [16].

Depending on the cell type, TFP counteracts oxidative stress by decreasing the formation of reactive oxygen species (ROS), thus preventing ROS-induced mitochondrial depolarization and protecting against apoptosis [17]. Along those lines TFP supports survival of T lymphocytes [1]. On the contrary, TFP may induce or augment apoptosis, especially of cancer cells [13, 14, 18, 19]. TFP inhibits cancer stem cell proliferation [12] and is effective against various malignancies including lung cancer [12-14], glioma [15], and leukemia [16]. Taken together, the effects of TFP on apoptotic pathways largely depend on the identity of the cell and/or on the cellular context.

In analogy to nucleated cell apoptosis, erythrocytes may enter eryptosis [20, 21], the suicidal death of erythrocytes characterized by cell shrinkage [22] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [20]. Eryptosis may be triggered by an increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\([20]\)). Further stimulators of eryptosis include ceramide [23, 24], oxidative stress [20], energy depletion [20], activated caspases [20, 25, 26], stimulated activity of casein kinase 1\(\alpha\), Janus-activated kinase JAK3, protein kinase Ca\(_\alpha\) p38 kinase and PAK2 kinase [20], impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [20] and/or exposure to a wide variety of xenobiotics [20, 27-41]. Inhibitors of eryptosis include erythropoietin [42] and nitric oxide [43].

The present study explored whether TFP induces eryptosis and, if so, to test whether the effect is sensitive to NO. Furthermore, the influence of a combination of extracellular Ca\(^{2+}\) removal and pre-treatment with the NO donor sodium nitroprusside (SNP) on eryptosis was studied.
Materials and Methods

Erythrocytes

Highly purified erythrocyte suspensions from healthy volunteers with white blood cell (WBC) or thrombocyte contaminations below 0.1 % [44] were provided by the blood bank of the University of Tübingen. Aliquots of the individual erythrocyte concentrates were either used directly at 0.4% hematocrit (Hct) or stored at 4°C for up to one week. The study was approved by the ethics committee of the University of Tübingen, the study was performed in agreement with the declaration of Helsinki, and volunteers gave written consent (184/2003 V).

Solutions and chemicals

Experiments analysing annexin-V-binding of erythrocytes and cell volume (0.4% Hct) as well as determination of cytosolic Ca\(^{2+}\) activity (0.1% Hct) were carried out in Ringer solution. Loading of erythrocytes (0.2% Hct) with 4 µM FLUO3/AM was performed in Fluo wash buffer. Staining of erythrocytes with annexin-V-FLUOS was performed in annexin binding buffer. Ringer solution was composed of (in mM): 125 NaCl, 5 KCl, 1.2 MgSO\(_4\), 32 N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, and 1 CaCl\(_2\). Annexin-binding buffer contained (in mM): 125 NaCl, 10 HEPES/NaOH (pH 7.4), and 5 CaCl\(_2\). Fluo loading buffer contained (in mM): 123 NaCl, 5 KCl, 1 MgSO\(_4\), 25 HEPES/NaOH (pH 7.4), 10 glucose, 2 CaCl\(_2\), and 10 sodium pyruvate. 50 mg trifluoperazine (10-[3-(4-methyl-1-piperazinyl)-propyl]-2-(trifluoromethyl)-(10H)-phenothiazine dihydrochloride, TFP) was dissolved in 10.4 ml DMSO to achieve a 10 mM stock solution. This stock was subsequently aliquoted in 250 µl units and stored at -20 °C for up to 3 months. Where indicated, trifluoperazine 2 HCl (TFP: 1 - 20 µM) was added. Dimethylsulfoxide (DMSO)-treated erythrocytes served as solvent controls (0.2% (v/v) DMSO). DMSO and sodium nitroprusside were purchased from Sigma (Taufkirchen, Germany). TFP and annexin V-FLUOS were purchased from Selleckchem (USA) and Roche Diagnostics (Mannheim, Germany), respectively. FLUO3/AM and CM-H\(_2\)DCFDA were purchased from Calbiochem (Germany) and Invitrogen (USA), respectively.

Phosphatidylserine exposure and forward scatter

Erythrocyte concentrates suspended in 2 ml Ringer solution (0.4% Hct) were treated in the absence or presence of TFP for 24 hours. After incubation under the respective experimental condition, 100 µl cell suspension (2·10\(^6\) erythrocytes) were washed in 500 µl annexin-binding buffer. Erythrocyte pellets were then vortexed gently to achieve a homogenous cell suspension. To detect phosphatidylserine (PS) on the outer leaflet of the plasma membrane, the cells were subsequently stained with 32 µl annexin V-FLUOS at a 1:33 dilution and mixed gently on a vortex mixer. After 20 min incubation in the dark at room temperature, 200 µl of annexin-binding buffer was added to each sample, thoroughly vortexed to achieve single cell suspensions, and analysed by flow cytometry on a FACS-Calibur (Becton Dickinson, Heidelberg, Germany). Erythrocyte volume was determined by forward scatter (FSC). To this end, corresponding erythrocytes suspensions were immediately analysed by flow cytometry.

Hemolysis

Erythrocyte concentrates (0.4% Hct) were treated with varying concentrations of TFP for 24 hours, then hemolysis was determined. Briefly, after incubation, 600 µl suspension containing 1.2·10\(^6\) erythrocytes were centrifuged for 4 min at 420 g, 4°C, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Measurement of cytosolic Ca\(^{2+}\)

Erythrocyte concentrates (0.2% Hct), suspended in 20 ml Fluo3 loading buffer were stained with Fluo3/AM at 4 µM final concentration. The cells were incubated at 37°C for 1 hour under shaking and protection from light. Excess dye was removed by washing erythrocytes twice with Fluo3 loading buffer and once with Ringer solution (6 min at 280 g, room temperature). For flow cytometry, Fluo3/AM-loaded erythrocytes (0.1% Hct) were resuspended in Ringer solution containing 0 – 20 µM TFP, or the Ca\(^{2+}\) ionophor ionomycin (0.25 µM) as a positive control, or vehicle alone (0.2% (v/v) as a negative control. After incubation for different time periods at 37 °C, 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.
**Fluorescence microscopy**

For fluorescence microscopy, Fluo4/AM-loaded erythrocytes (0.1% Hct) suspended in Ringer solution were treated in the absence or presence of TFP for 1 hour, 3 hours, or 6 hours. The preparation of RBCs for microscopy experiments were the same as for flow cytometry measurements. Erythrocytes were monitored using an inverted fluorescence microscope (Eclipse TE2000-E, Nikon, Tokyo, Japan) as described before [45, 46]. The diluted erythrocyte samples (approximately 0.025% haematocrit) were placed on a cover slip in a dark room at room temperature. Images were taken with an electron multiplication CCD camera (CCD97, Photometrics, Tucson, USA) using a 100x1.4 (NA) oil immersion lens with infinity corrected optics. From each RBC sample, 5 images from different positions of the cover slip randomly chosen were taken using the imaging software VisiView (Visitron Systems, Puchheim, Germany). Each image consisted of one transmitted light (exposure time 200 ms) and one fluorescence shot (exposure time 4 s). Fluo-4 (instead of Fluo-3) has been used for Ca\(^{2+}\) imaging and was excited with a xenon lamp-based monochromator (Visitron Systems, Puchheim, Germany) at a centre wavelength of 488 nm. Emission was recorded at 520/15 nm. Fluo-4 AM was obtained from Molecular Probes (Eugene, USA).

**Patch clamp experiments**

Patch-clamp measurements were performed with a NPC-16 Patchliner (Nanion Technologies, Munich, Germany) [47]. The resistance of the chips was between 5 and 8 MΩ with internal and external solutions as follows (in mM): KCl 70, KF 70, NaCl 10, HEPES 10, MgATP 2, EGTA 3 and CaCl\(_2\) 1.2 to give 120 nM free [Ca\(^{2+}\)]\(_i\), pH=7.2 adjusted with KOH (internal) and NaCl 140, KCl 70, MgCl\(_2\) 5, D-glucose 5, HEPES 10, CaCl\(_2\) 2, pH=7.3 adjusted with NaOH (external). Gigaseals were considered successful if exceeding 5 GΩ (with most cells they were 10 GΩ and above). Gigaseal formation was facilitated by the use of a seal enhancing solution as recommended by the Patchliner manufacturer and containing (in mM): NaCl 80, KCl 3, MgCl\(_2\) 10, CaCl\(_2\) 35, HEPES 10, pH=7.3 adjusted with NaOH. Whole-cell configuration was achieved by negative pressure suction pulses between -45 mbar and -150 mbar and its formation judged by the appearance of sharp capacitive transients. Whole-cell patch-clamp recordings were conducted at room temperature using voltage steps from -100 mV to 80 mV for 500 ms in 20 mV increments at 5 s intervals, the holding potential being set at -30 mV. Assessment of the effect of TFP was carried out with 1 and 10 µM. To reduce inter-cell variability data are expressed as normalized current which is the ratio of the current under specified experimental conditions i.e. before (control) and in the presence of 1 and 10 µM TFP at selected membrane potentials, to the current at +80 mV determined 30–60 s before starting the control (control) measurement.

**Fluorescence switch experiments for assessing S-nitrosylation**

RBC concentrates (0.4% Hct) were treated with varying concentrations of TFP for 24 hours, with some samples pretreated with sodium nitroprusside (SNP) for 2 hours, and subjected to the fluorescence switch assay as previously described [48]; in brief, cells were lysed and blocked with N-ethylmaleimide, nitrosothiols reduced with ascorbate and newly formed thiols labelled with maleimide-Bodipy-TMR (Molecular Probes). A methodological control was performed by adding 1 mM S-nitroso-L-cysteine 15 min prior to erythrocyte lysis. Protein extracts subjected to the fluorescence switch were separated by SDS-PAGE, Bodipy-TMR signal was registered and the gels were stained with Sypro Ruby (Molecular Probes), following manufacturer’s instructions, for assessing total protein in each lane. Both signals were quantified using ImageQuant TL v7.0 software (GE Healthcare) and the ratio S-nitrosylation/total protein in each sample estimated from the Bodipy-TMR/Sypro Ruby ratio.

**Statistical analysis**

Data are presented as the mean values ± SEM of at least 3 independent experiments with different blood samples. A total of 23 different blood samples were used in this study. Unless otherwise stated, repeated measures one-way ANOVA with Dunnet’s post test was used for statistical comparisons of treated samples with controls. Differences of the means were considered to be statistically significant when the calculated p value was *P<0.05, **P<0.01, ***P<0.001, or ****P<0.0001.
Results

Induction of eryptosis and cell shrinkage by trifluoperazine

The present study explored whether trifluoperazine (TFP) stimulates eryptosis, the suicidal erythrocyte death characterized by phosphatidylserine translocation to the cell surface, cell shrinkage and increased Ca\(^{2+}\) influx and, if so, to test whether these effects could be reversed by the nitric oxide donor sodium nitroprusside (SNP).

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding and flow cytometry. Prior to measurements, erythrocytes were incubated for 24 hours in Ringer solution in the presence or absence of TFP (1 – 20 µM). As shown in Fig. 1A, B, exposure to TFP concentration-dependently increased the percentage of phosphatidylserine-exposing erythrocytes, an effect reaching statistical significance at 2.5 µM TFP. To study hemolytic effects of TFP, we determined the hemoglobin concentration in the supernatant of drug-treated erythrocytes. As illustrated in Fig. 1C, exposure to TFP increased the percentage of hemolytic erythrocytes. At 20 µM TFP, the hemolysis reached 11% after 24 hours (Fig. 1C).

An extended exposure (24 hours) to 10 µM TFP decreased significantly the average erythrocyte forward scatter whereas incubation of the cells with 5 µM TFP resulted in significant swelling when compared to DMSO-treated erythrocytes (Fig. 1D, E).

Impact of TFP on cytoplasmic Ca\(^{2+}\) concentration

Fluo3 fluorescence was taken as a measure of cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). As shown in Fig. 2A, B, exposure to TFP increased the percentage of erythrocytes with enhanced Fluo3 fluorescence, an effect reaching statistical significance after 1 hour at 1 µM TFP. At the highest concentration of TFP (20 µM), intracellular Ca\(^{2+}\) concentration...
was equal to the maximum calcium load induced by the Ca\(^{2+}\) ionophore ionomycin (0.25 µM; Fig. 2A, B). A short exposure (1 – 6 hours) to low concentrations of TFP increased the average erythrocyte forward scatter pointing to cell swelling, an effect reaching statistical significance at 5 – 15 µM TFP (Fig. 2C). For comparison, the effect of ionomycin (0.25 µM) on Fluo 3 fluorescence and forward scatter (FSC) of the erythrocyte populations is shown (Fig. 2B, C, black bars).

Fluorescence microscopy experiments, using Fluo 4/AM confirmed the ability of TFP to increase the intracellular Ca\(^{2+}\) concentration in erythrocytes after 1 – 6 hours exposure time. In these experiments, DMSO- as well as TFP-treated erythrocytes showed a normal disc-like shape (Fig. 3A,B). A magnification of the fluorescence image of two erythrocytes treated with 10 µM TFP for 6 hours is illustrated in Figure 3B.

**Effect of TFP on whole-cell currents**

Patch-clamp measurements of whole-cell currents were performed in the presence of 1 and 10 µM TFP. Fig. 4 shows the averaged I/V curves of control measurements and after application of 1 µM TFP (Fig. 4A) or 10 µM TFP (Fig. 4B). While 1 µM TFP did not have any effect on either the inward or outward current, 10 µM TFP significantly decreased the outward current.

**TFP-induced eryptosis is inhibited by exogenous NO, an effect augmented by simultaneous removal of extracellular calcium**

A next series of experiments explored whether the TFP-induced cell shrinkage and translocation of phosphatidylserine required entry of extracellular Ca\(^{2+}\). To this end, erythrocytes were incubated for 24 hours in the absence or presence of 1, 5, and 10 µM trifluoperazine in the presence or nominal absence of extracellular Ca\(^{2+}\). As illustrated in Fig. 5A, removal of extracellular Ca\(^{2+}\) even in the presence of 1 mM EGTA did not influence the effect of TFP on the percentage of annexin-V-binding erythrocytes, pointing to a Ca\(^{2+}\)-independent induction of eryptosis by TFP. To explore whether TFP-induced eryptosis was sensitive to NO, erythrocytes suspended in Ringer solution were first pre-treated with the NO donor SNP (1 µM) for 2 hours. Subsequently, the cells were either treated with different TFP concentrations (1 - 10 µM) or with DMSO for further 24 hours. TFP-induced
eryptosis was significantly inhibited by SNP in the presence (Fig. 5B) as well as absence (Fig. 5C) of calcium. The inhibitory effect was significantly more pronounced by a combination of removal of extracellular Ca\(^{2+}\) and pre-treatment with SNP (Fig. 5D). Compared with the NO-donor SNP (1 µM), addition of the anti-oxidant N-acetyl-L-cysteine (NAC, 1mM) showed less inhibitory effect on TFP-induced annexin-V-binding (Fig. 5E).

**Blunting of ionomycin induced eryptosis and cell shrinkage by removal of extracellular Ca\(^{2+}\)**

As positive control, erythrocytes were incubated in Ringer solution or in Ringer solutions without extracellular Ca\(^{2+}\) ±1 mM EGTA. As expected, ionomycin-induced eryptosis and cell shrinkage was completely abrogated by removal of extracellular Ca\(^{2+}\) without or with addition of 1 mM EGTA (Fig. 6).

**Reversal of TFP induced inhibition of S-nitrosylation by addition of NO donor sodium nitroprusside (SNP)**

One of the non-classical mechanisms of NO signaling proceeds through the formation of S-nitrosylation, a covalent modification of protein cysteine residues [49, 50]. Protein S-nitrosylation can be detected by specific fluorescent labelling with a “fluorescence switch” procedure [48, 51]. We therefore used this technique to evaluate the extent of S-nitrosylation in erythrocytes (Fig. 7A). TFP treatment for 24 hours decreased protein S-nitrosylation, with a significant effect observed at concentrations higher than 2.5 µM (Fig. 7B). We further
investigated whether the NO donor SNP could reverse the effect of TFP on S-nitrosylation. SNP pretreatment dissipated the inhibitory effect of 10 µM TFP on S-nitrosylation (Fig. 7B).

**Discussion**

The present observations reveal that trifluoperazine (TFP) triggers cell membrane scrambling, as apparent from phosphatidylserine exposure and erythrocyte shrinkage. Moreover, TFP triggers hemolysis as evident from increased turbidity of the supernatants harvested from the corresponding erythrocyte suspensions (Fig. 1 A-C). However, somehow surprisingly, incubation with a lower concentration of TFP (5 µM) for short (1 – 6 hours) as well as for long time periods (24 hours) resulted in significant erythrocyte swelling (Fig. 2C and Fig. 1D, E). The mechanism accounting for this cell swelling remains elusive. Putative mechanisms causing cell swelling include inhibition of K⁺ channels [52]. Along those lines, TFP has been shown to inhibit a variety of K⁺ channels [53-57]. Cell swelling may in turn affect cytoskeletal architecture [58, 59].

The effect of TFP on cell membrane scrambling was paralleled by an increase of cytoplasmic Ca²⁺ (Fig. 2A, B and Fig. 3A, B). The effect of 1 µM TFP was small but statistically
significant. Although Ca^{2+} is a well known trigger of cell membrane scrambling [20], TFP-induced phosphatidylserine translocation was not affected by removal of extracellular Ca^{2+} or by addition of 1 mM EGTA to Ringer solution without extracellular Ca^{2+} (Fig. 5A) suggesting a Ca^{2+}-independent mechanism triggering phosphatidylserine exposure on the outer membrane leaflet [45]. In contrast, ionomycin-induced eryptosis is expectedly [60] calcium-dependent (Fig. 6A,B). The patch-clamp measurements revealed that TFP failed to increase the inward
Fig. 8. Graphical representation of the study’s central findings. I) Eryptosis is mediated by TFP in a Ca²⁺-dependent and a Ca²⁺-independent manner. II) Pre-treatment of erythrocytes with the NO donor SNP (1 µM) significantly inhibits TFP-induced eryptosis. III) The inhibitory effect of SNP is augmented by removal of extracellular Ca²⁺. Abbreviations: Trifluoperazine (TFP), Calcium (Ca²⁺), Nitric Oxide (NO), Sodium Nitroprusside (SNP).

current (Fig. 4). Those experiments do, however, not rule out the opening of low conductance ion channels. Alternatively, the observed increase of cytosolic Ca²⁺ concentration may result from an inhibitory effect of TFP on calmodulin [61] which is required for the operation of the erythrocytes’ plasma membrane Ca²⁺ pump [62]. Calmodulin antagonism could also account for the observed decrease in the outward current as TFP may block Ca²⁺-stimulated K⁺ transport in erythrocytes, possibly the Gardos channel, in the concentration range used in our study [63]. However, although it seems likely that some of the observed effects of TFP result from calmodulin antagonism, the fact that eryptotic events are virtually abrogated by a NO donor (nitroprusside) points to an inhibitory effect of TFP on NO synthase. Along those lines, TFP diminishes the S-nitrosylation levels (Fig. 7). Erythrocytes possess a functional NO synthase and are a major source of NO, contributing to the circulating NO pool [64]. In addition, NO synthase inhibitors reduce erythrocyte deformability [65].

TFP further leads to moderate hemolysis. The physiological function of eryptosis is the clearance of defective erythrocytes from circulating blood prior to hemolysis [20]. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. Timely removal of defective erythrocytes prevents release of hemoglobin, which may cross the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus cause renal failure [66]. In malaria, eryptosis may accomplish elimination of infected erythrocytes thus limiting parasitemia [67].

The clearance of eryptotic erythrocytes from circulating blood may, however, lead to anemia, if the loss of eryptotic erythrocytes exceeds the formation of new erythrocytes by erythropoiesis [20]. Phosphatidylserine exposing erythrocytes may further adhere to the vascular wall [68], stimulate blood clotting [69] and trigger thrombosis [70], effects potentially compromising microcirculation [23, 71, 72]. Excessive eryptosis may thus contribute to anemia and jeopardise microcirculation in several clinical conditions, including iron deficiency [20], dehydration [73], hyperphosphatemia [74], chronic kidney disease (CKD) [75, 76], hemolytic-uremic syndrome [77], diabetes [78], hepatic failure [79], malignancy [20], sepsis [80], sickle-cell disease [20], beta-thalassemia [20], Hb-C and G6PD-deficiency [20], as well as Wilsons disease [81]. In those conditions the eryptotic effect of TFP may be particularly threatening.

One of the central findings of this study points to antagonistic roles of Ca²⁺ and NO. For the first time, we show that pre-treatment of erythrocytes with the NO donor SNP (1 µM) can significantly inhibit TFP-induced eryptosis, and that this inhibitory effect is augmented by additional removal of extracellular Ca²⁺. Accordingly, a Ca²⁺-dependent mechanism counter-regulates the effect of NO.

Plasma levels of free or bound NO are in the 3 nM or 7 µM range, respectively [82]. However, the blood plasma levels of free Ca²⁺ are within the 1 to 1.5 mM range. There is a strong correlation between the elevation of [Ca²⁺], and ROS formation, two cross-talking messengers capable to initiate apoptosis [83]. Interestingly, ROS and in particular superoxide anion reacts with NO diminishing its bioavailability [84]. Thus, TFP-induced accumulation of [Ca²⁺], (Fig. 2A, B and Fig. 3A, B) could lead to ROS formation, subsequent destruction of NO and thus induction of eryptosis. In the presence of Ca²⁺ calmodulin avidly binds to two TFP molecules and thus forces its own inactivation (for review see [85]). Along those lines, TFP-induced accumulation of [Ca²⁺], serves to biologically inactivate calmodulin, thus inducing eryptosis. It is known that calmodulin inhibitors induce apoptosis [86]. On the other hand,
we also observed a Ca\(^{2+}\)-independent induction of eryptosis by TFP (Fig. 5A) which was efficiently inhibited by the addition of exogenous NO (Fig. 5B – C and Fig. 8).

SNP addition did not increase S-nitrosylation levels, but reversed the effect of TFP on S-nitrosylation (Fig. 7). Possibly, in the absence of TFP, the S-nitrosylation is already maximal and cannot be increased further.

In conclusion, TFP triggers eryptosis in a Ca\(^{2+}\)-dependent and a Ca\(^{2+}\)-independent manner, effects paralleled by a decrease in S-nitrosylation, and sensitive to the inhibitory effect of nitric oxide pretreatment. Furthermore, antagonistic roles of Ca\(^{2+}\) and NO, shown in this study, should deepen our knowledge about calcium- and nitric oxide-associated diseases.

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Disclosure Statement

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