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Original Paper

Tanshinone IIA Stimulates Erythrocyte Phosphatidylserine Exposure

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Key Words
Phosphatidylserine • Cell membrane scrambling • Calcium • Cell volume • Eryptosis

Abstract
Tanshinone IIA, an antimicrobial, antioxidant, antianaphylactic, antifibrotic, vasodilating, antiatherosclerotic, organo-protective and antineoplastic component from the rhizome of *Salvia miltiorrhiza*, is known to trigger apoptosis of tumor cells. Tanshinone IIA is effective in part through mitochondrial depolarization and altered gene expression. Erythrocytes lack mitochondria and nuclei but may undergo eryptosis, an apoptosis-like suicidal cell death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Eryptosis is triggered by increase of cytosolic Ca^{2+} activity, ATP depletion and ceramide formation. The present study explored, whether tanshinone IIA elicits eryptosis. Cytosolic Ca^{2+}-concentration was determined from Fluo3-fluorescence, cell volume from forward scatter, phosphatidylserine exposure from binding of fluorescent annexin V, hemolysis from hemoglobin concentration in the supernatant, ATP concentration utilizing luciferin–luciferase and ceramide formation utilizing fluorescent anticeramide antibodies. Clearance of circulating erythrocytes was estimated by CFSE-labeling. A 48 h exposure to tanshinone IIA (≥10 μM) significantly increased cytosolic Ca^{2+}-concentration, decreased ATP concentration (25 μM), increased lactate concentration (25 μM), increased ceramide formation (25 μM), decreased forward scatter, increased annexin-V-binding and increased (albeit to a much smaller extent) hemolysis. The effect of 25 μM tanshinone IIA on annexin-V binding was partially reversed in the nominal absence of Ca^{2+}. Labelled tanshinone IIA-treated erythrocytes were more rapidly cleared from the circulating blood in comparison to untreated erythrocytes. The present observations reveal a completely novel effect of tanshinone IIA, i.e. triggering of Ca^{2+} entry, ATP depletion and ceramide formation in erythrocytes, events eventually leading to eryptosis with cell shrinkage and cell membrane scrambling.

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Introduction

Tanshinone IIA (MW 294) [1] from the rhizome of *Salvia miltiorrhiza* [2, 3] is an active ingredient of Danshen, a well-known traditional Chinese medicine used for multiple therapeutic purposes [3] including cardiovascular diseases, such as coronary heart disease and stroke [4-6] as well as antitumor activity [7]. Efficacy of tanshinone IIA has been demonstrated both *in vitro* and *in vivo* [3, 8].

Tanshinone possesses antimicrobial [9, 10], antioxidant [11, 12], antianaphylactic [13], antifibrotic [14], vascular [5, 15, 16], antiatherosclerotic [17], cardioprotective [11, 12, 18, 19], pulmonary protective [20-22], renoprotective [23], bone protective [24], neuroprotective [12, 25-27] and antineoplastic [28-31] activities.

The antineoplastic effect of tanshinone IIA results from induction of apoptosis, as shown in cells derived from ovarian cancer [29], prostate cancer [28, 30] and acute promyelocytic leukemia [31]. Beyond that tanshinone may induce hepatic stellate cell apoptosis [32] On the other hand, tanshinone IIA has been shown to inhibit apoptosis of cardiomyocytes [11, 33]. The effect of tanshinone IIA involves mitochondria [34], altered gene expression [35], Ca^{2+} mobilisation and Ca^{2+} influx [36].

Similar to apoptosis of nucleated cells, eryptosis, the suicidal death of erythrocytes, leads to cell membrane scrambling and cell shrinkage [37]. As erythrocytes lack mitochondria and nuclei, eryptosis is independent from mitochondrial dysfunction and altered gene expression. Instead, eryptosis is triggered by Ca^{2+} entry through Ca^{2+}-permeable cation channels [38, 39]. Ca^{2+} activates Ca^{2+}-sensitive K^+ channels [40] with subsequent exit of KCl together with osmotically obliged water and thus cell shrinkage [41]. In addition, Ca^{2+} triggers cell membrane scrambling with subsequent exposure of phosphatidylserine at the cell surface [42]. Ca^{2+} further triggers cell membrane scrambling [37]. The cell is sensitized to the eryptotic effects of Ca^{2+} by ceramide [43], which is generated by acid sphingomyelinase [44]. Eryptosis is further triggered by energy depletion [45]. Moreover, erythrocyte cell membrane scrambling may be triggered by caspases [46, 47], which are activated by oxidative stress but are not required for the effect of Ca^{2+} on cell membrane scrambling [42]. Erythrocyte survival may further be modulated by kinases involved in the signaling of apoptosis, such as p38 MAPK and CK1 [48, 49].

The present study explored, whether tanshinone IIA influences cytosolic Ca^{2+} activity, cell volume and cell membrane scrambling of human erythrocytes and thus the programmed cell death of erythrocytes.

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 with or without 1, 10 or 25 μM tanshinone IIA (purity > 98%/TLC, Enzo Alexis). Where indicated, extracellular glucose was removed or tanshinone IIA (Enzo, Lörrach, Germany) added at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted for 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 were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with fluorescein-isothiocyanate (FITC)-conjugated Annexin-V (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+}

After incubation 50 µl suspension 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 in FACS analysis.

Determination of intracellular ATP concentration

For determination of intracellular erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without tanshinone IIA (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

Determination of lactate generation

For the determination of lactate formation, 200 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without 25 µM tanshinone IIA (final hematocrit 20%). After 48 h, the samples were centrifuged (3 min at 400 g, room temperature) and the supernatant was collected. Total lactic acid content in the supernatant was measured by a commercial lactate assay kit (Bioassay systems) according to the manufacturer's instructions.

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without tanshinone IIA, 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 analysed by flow cytometric analysis in FL-1. As shown previously [44], the monoclonal antibody-based assay yields similar results as the biochemical determination of ceramide utilizing a diacylglycerol-kinase assay (Amersham Biosciences).

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.

Confocal microscopy and immunofluorescence

For the visualisation of eryptotic erythrocytes, 4 µl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated Annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally resuspended in 50 µl of Ringer solution containing 5 mM $CaCl_2$. 20 µl were mounted with Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) 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 the in vivo clearance of fluorescence-labeled erythrocytes

Following treatment with or without tanshinone IIA, erythrocytes (obtained from 200 µl blood) were fluorescence-labeled by staining with 5 µM carboxyfluorescein-diacetate-succinimidyl-ester (CFSE) (Molecular Probes, Leiden, Netherlands) in PBS and incubated for 30 min at 37°C. After washing twice in PBS containing 1% FCS the pellet was resuspended in Ringer solution (37°C), and 100 µl of the CFSE-labeled erythrocytes were injected into the tail vein of the recipient mouse. After 36 h, blood was retrieved from the tail veins of the mice, and CFSE-dependent fluorescence intensity of the erythrocytes was measured in FL-1 as described above. The percentage of CFSE-positive erythrocytes was calculated in % of the total labeled fraction determined 5 min after injection.

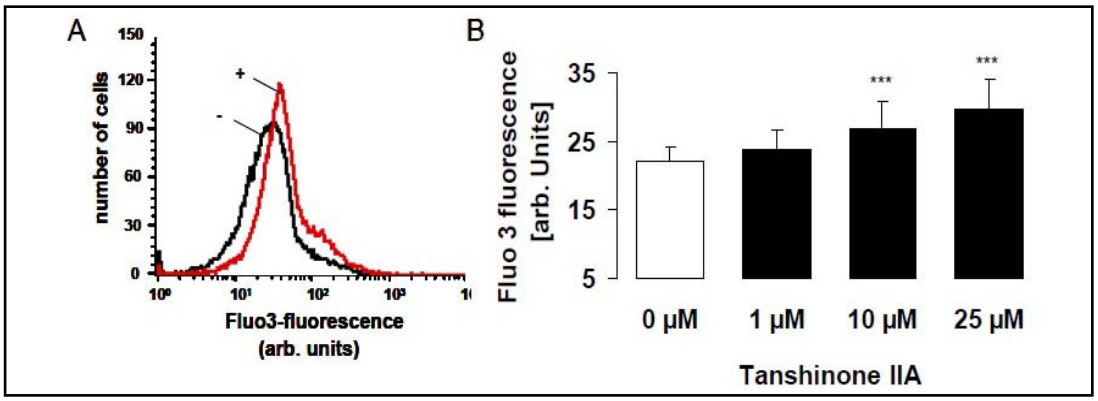

Fig. 1. Effect of tanshinone IIA on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 25 µM tanshinone IIA. B. Arithmetic means ± SD (n = 13) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 -fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) 1-25 µM tanshinone IIA. *** ($p < 0.001$) indicates significant difference from the absence of tanshinone IIA (paired ANOVA).

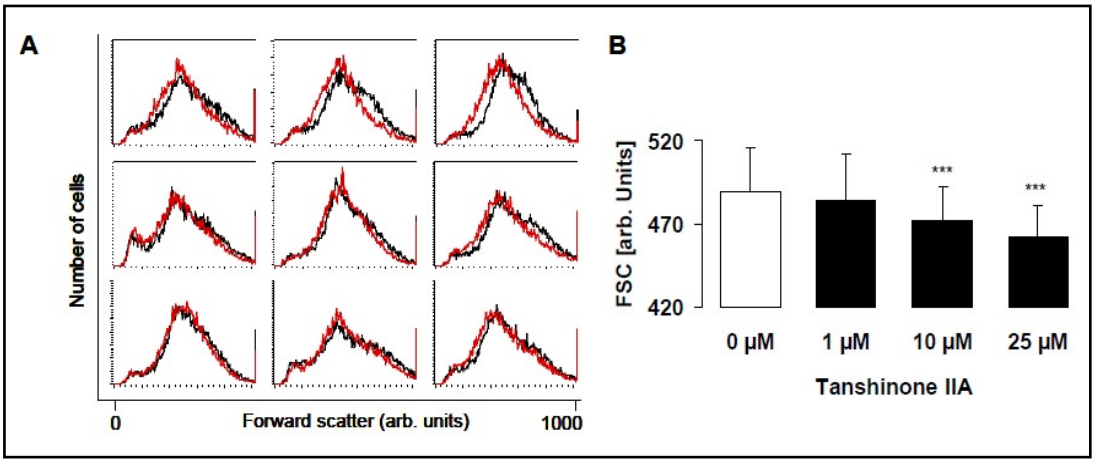

Fig. 2. Effect of tanshinone IIA on erythrocyte forward scatter. A. Original histograms of forward scatter of erythrocytes from 9 patients following exposure for 48 h to Ringer solution without (black lines) and with (red lines) presence of 25 µM tanshinone IIA. B. Arithmetic means ± SD (n = 13) of the erythrocyte forward scatter following incubation for 48 h to Ringer solution without (white bar) or with (black bars) 1-25 µM tanshinone IIA. *** ($p < 0.001$) indicates significant difference from the absence of tanshinone IIA (paired ANOVA).

Statistics

Data are expressed as arithmetic means ± SD. Statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptotic effects, the same erythrocyte specimens have been used for control and experimental conditions.

Results

To determine cytosolic Ca^{2+} concentration, Fluo 3-fluorescence was determined in FACS analysis. As shown in Fig. 1, treatment of human erythrocytes with tanshinone IIA resulted in an increase of Fluo3-fluorescence reflecting an increase of cytosolic Ca^{2+} concentration. The effect of tanshinone IIA on Fluo3-fluorescence reached statistical significance at a tanshinone IIA concentration of 10 µM.

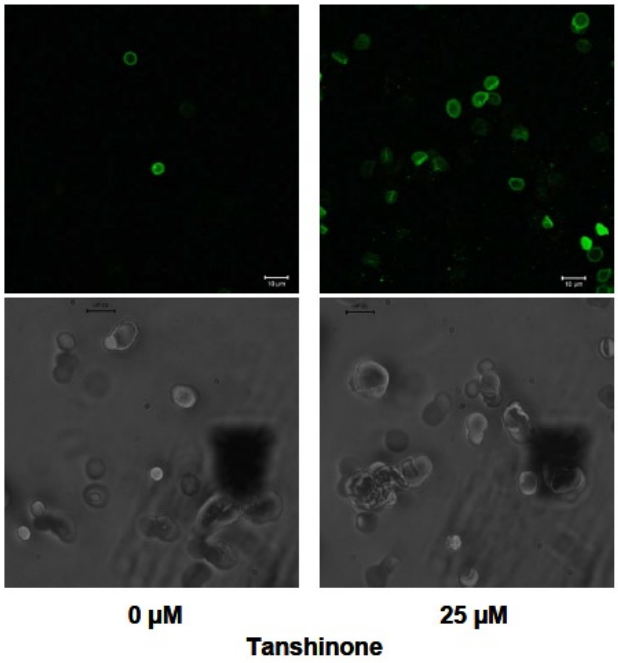

Fig. 3. Confocal images of PS-exposing erythrocytes with or without tanshinone IIA treatment. Confocal microscopy of FITC-dependent fluorescence (upper panels) and light microscopy (lower panels) of human erythrocytes stained with FITC-conjugated Annexin-V Fluos following 48 h incubation in Ringer solution without (left panels) and with (right panels) 25 µM tanshinone IIA.

Ca^{2+} is known to activate erythrocyte Ca^{2+} sensitive K^+ channels and an increase of cytosolic Ca^{2+} concentration is expected to trigger exit of KCl which, osmotically, obliges water to follow, an effect resulting in cell shrinkage. To determine the effect of tanshinone IIA on cell volume, forward scatter was determined in FACS analysis. As illustrated in Fig. 2, tanshinone IIA treatment was indeed followed by a decrease of forward scatter. The effect of tanshinone IIA on forward scatter reached statistical significance at a tanshinone IIA concentration of 10 µM.

Ca^{2+} is further known to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin -V-binding. The fluorescent annexin-V-positive erythrocytes were visualized by confocal imaging. As shown in Fig. 3, a 48 h exposure to 25 µM tanshinone IIA was followed by the appearance of annexin-V-positive erythrocytes, an observation pointing to the triggering of cell membrane scrambling.

Quantification of annexin-V-binding was accomplished by FACS analysis. As demonstrated in Fig. 4A and 4B, a 48 h treatment with tanshinone IIA increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 10 µM tanshinone IIA.

For determination of hemolysis hemoglobin release into the supernatant was quantified in erythrocytes exposed for 48 h to Ringer solution without or with 1-25 µM tanshinone IIA. As illustrated in Fig. 4B, tanshinone IIA treatment was followed by hemolysis, an effect reaching statistical significance at 10 µM tanshinone IIA. The percentage of hemolytic erythrocytes remained, however, by far smaller than the percentage of annexin -V-binding erythrocytes.

Further experiments aimed to define the causal role of Ca^{2+} in the triggering of cell membrane scrambling by tanshinone IIA. Erythrocytes were exposed to tanshinone IIA either in the presence or in the nominal absence of extracellular Ca^{2+}. As shown in Fig. 4C, the effect of tanshinone IIA on annexin-V-binding was blunted, but not fully abolished in the nominal absence of Ca^{2+}. Instead, the annexin-V-binding was in the nominal absence of Ca^{2+} but presence of tanshinone IIA significantly lower than the respective value in the presence of both Ca^{2+} and tanshinone IIA, but at the same time significantly higher than the respective value in the absence of tanshinone IIA in both, the presence or absence, of Ca^{2+}. The effect of tanshinone IIA on cell membrane scrambling is thus in part due to an increase of intracellular Ca^{2+} activity.

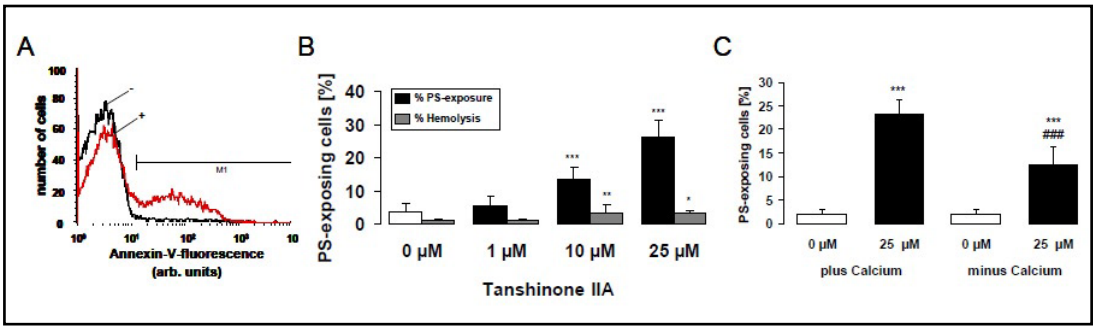

Fig. 4. Effect of tanshinone IIA on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 25 µM tanshinone IIA. B. Arithmetic means ± SD (n = 13) of erythrocyte annexin- V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of 1-25 µM tanshinone IIA. For comparison, arithmetic means ± SD (n = 7) of the percentage of hemolysis is shown as grey bars. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from the absence of tanshinone IIA (paired ANOVA). C. Arithmetic means ± 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) 25 µM tanshinone IIA in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** ($p < 0.001$) indicates significant difference from the absence of tanshinone IIA (paired ANOVA), ### ($p < 0.001$) indicates significant difference from the respective values in the presence of Ca^{2+}.

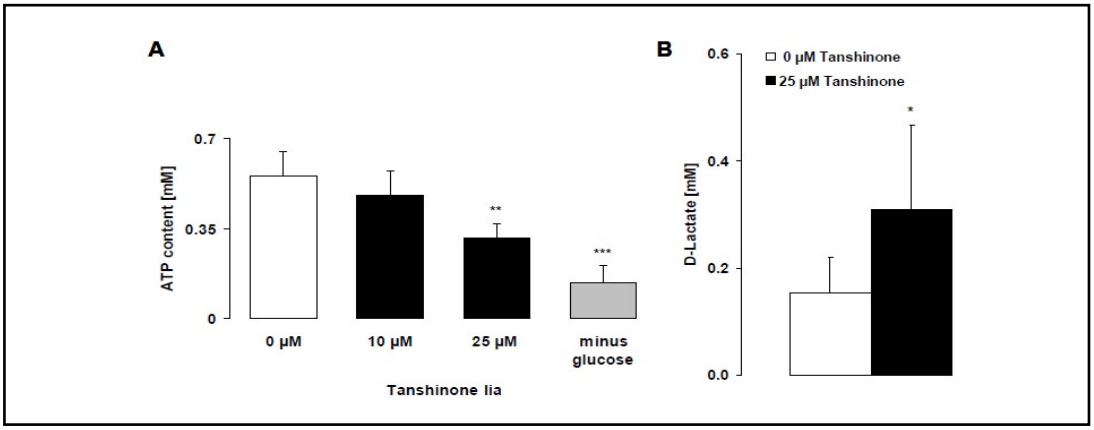

Fig. 5. Effect of tanshinone IIA on erythrocyte cytosolic ATP content and lactic acid release. A. Arithmetic means ± SD (n = 4) of the ATP concentration after a 48 h incubation in Ringer solution without (white bar) or with (black bars) tanshinone IIA at the indicated concentrations, or in glucose-depleted Ringer solution (grey bar, minus glucose). ** ($p < 0.01$), *** ($p < 0.001$) indicates significant difference from control (absence of tanshinone IIA and presence of glucose) (paired ANOVA). B. Tanshinone IIA-sensitive lactic acid formation in erythrocytes. Arithmetic means ± SD (n= 6) of lactic acid formation in erythrocytes following incubation for 48 h in the absence (white bar) or presence (black bar) of 25 µM tanshinone IIA. * ($p < 0.05$) indicates significant difference from the absence of tanshinone II A (paired two-tailed t-test).

Further experiments were performed to possibly identify further mechanisms underlying the stimulating effect of tanshinone IIA on cell membrane scrambling. Known triggers of eryptosis include energy depletion. Accordingly, the effect of tanshinone IIA treatment on cytosolic ATP concentration was determined. As illustrated in Fig. 5a, the cytosolic ATP concentration was significantly lower in erythrocytes incubated for 48 h in Ringer containing tanshinone IIA than in erythrocytes exposed for 48 h in Ringer without tanshinone IIA. The effect of tanshinone IIA reached statistical significance at 25 µM tanshinone IIA concentration. For comparison, the ATP concentration was determined in erythrocytes exposed to glucose-free Ringer. As shown in Fig. 5a, glucose depletion was followed by a more profound decrease of ATP concentration than the treatment with tanshinone IIA.

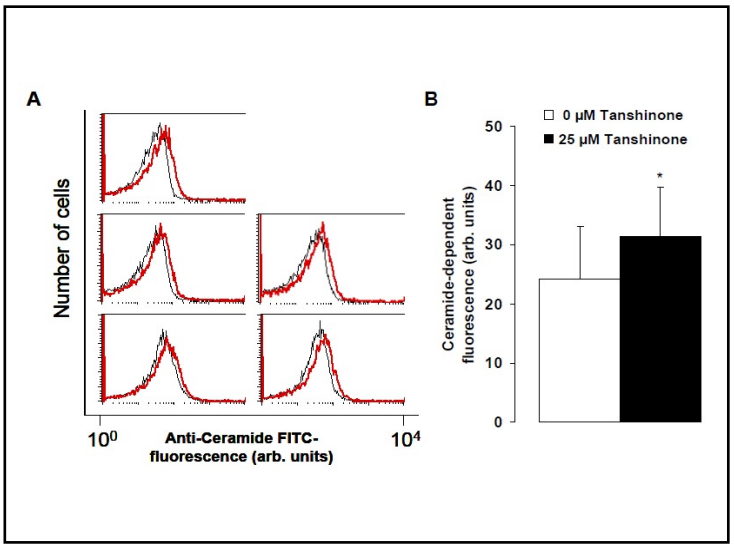

Fig. 6. Effect of tanshinone IIA on ceramide formation. A. Original histograms of ceramide abundance in erythrocytes from 5 patients following exposure for 48 h to Ringer solution without (black lines) and with (red lines) presence of 25 µM tanshinone IIA. B. Arithmetic means ± SD (n = 5) of ceramide abundance in erythrocytes following incubation for 48 h to Ringer solution without (white bar) or with (black bar) presence of 25 µM tanshinone IIA. * ($p < 0.05$) indicates significant difference from the absence of tanshinone IIA (paired ANOVA).

As the cytosolic ATP concentration was decreased by tanshinone IIA, the production of lactic acid was examined. As shown in Fig. 5b, the concentration of lactic acid in the supernatant of erythrocytes incubated for 48 h in Ringer containing 25 µM tanshinone IIA was significantly higher than in erythrocytes exposed for 48 h in Ringer without tanshinone IIA.

Cell membrane scrambling is further known to be triggered by ceramide. Accordingly, FITC-labelled anticeramide antibodies were employed to analyse the effect of tanshinone IIA on ceramide abundance in the erythrocyte cell membrane. As shown in Fig. 6, tanshinone IIA (25 µM) treatment indeed significantly increased ceramide abundance indicating that tanshinone IIA stimulates ceramide formation.

To elucidate whether tanshinone IIA influenced the *in vivo* life span of erythrocytes, erythrocytes were isolated from blood drawn from wild type mice, treated with tanshinone IIA (25 µM) for12 h, labeled with the fluorescent dye CFSE, and subsequently injected into the tail vein of the same mice. The clearance of CFSE-labeled erythrocytes from the circulation was determined 36 h after reinjection. As a result tanshinone II A treatment significantly ($p < 0.05$) accelerated the clearance of erythrocytes from circulating blood. The percentage of CSFE labelled erythrocytes remaining in circulating blood after 36 h was 56 ± 15 % (n = 3) without treatment and 13 ± 1 % (n = 3) with tanshinone IIA treatment.

Discussion

The present observations reveal a completely novel effect of tanshinone IIA, i.e. the stimulation suicidal erythrocyte death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage. The concentrations triggering eryptosis are within the range of tanshinone IIA plasma concentrations presumably approached *in vivo*. In mice, a dosages of 3 - 100 mg/kg tanshinone IIA have been administrated [12, 22, 50-55]. Following the application of 8 mg/kg, the plasma concentration increased up to some 3 µM [1]. The eryptosis is paralleled by hemolysis, which affects, however, only a comparably small percentage of erythrocytes.

The effect of tanshinone IIA on cell membrane scrambling is partially due to increase of cytosolic Ca^{2+} activity, which is known to trigger erythrocyte membrane scrambling [42, 46, 56]. According to indirect evidence, tanshinone IIA rather decreases Ca^{2+} entry into vascular smooth muscle cells. The channels involved remained elusive. In erythrocytes, Ca^{2+} entry presumably involves TRPC6 [38].

The incomplete inhibition of tanshinone IIA -induced cell membrane scrambling under Ca^{2+}-depleted conditions prompted us to search for the effect of tanshinone on further mechanisms known to trigger eryptosis. Those mechanisms include energy depletion [45]. As a result, tanshinone IIA indeed decreases cytosolic ATP concentration. This effect is again moderate and contributes to but does not fully account for the strong stimulation of cell membrane scrambling. Erythrocyte ATP generation is dependent on glycolysis and the effect of tanshinone IIA on cytosolic ATP levels could have reflected an interference with glycolytic flux. Tumor cells gain their energy mainly from glucose degradation [57] and in theory impaired glycolysis could contribute to the known [28-31, 58] antineoplastic effect of tanshinone. However, lactic acid production was increased following incubation with 25 μM tanshinone IIA. Thus, glycolytic flux appears to be enhanced by tanshinone IIA treatment and the substance presumably decreases cytosolic ATP levels by increasing ATP utilization.

Cell membrane scrambling is further stimulated by ceramide [43, 59]. Again, tanshinone IIA moderately increases ceramide abundance, an effect contributing to but not fully accounting for the stimulation of cell membrane scrambling.

Beyond its effect of cell membrane scrambling Ca^{2+} activates Ca^{2+} sensitive K^+ channels [40, 60] with resulting K^+ exit, cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of water [41]. Tanshinone IIA indeed decreases the forward scatter, an observation pointing to cell shrinkage. In nucleated cells tanshinone has been shown to activate several types of K^+ channels [16, 20, 61], which would similarly hyperpolarize the cell membrane, increase the electrical driving force for Cl^- exit and thus result in cellular KCl loss. On the other hand, tanshinone has been shown to downregulate aquaporins [21], which would impede water fluxes and thus cell volume changes.

Signaling involved in the effects of tanshinone IIA in nucleated cells further includes Akt-GSK-3β [19, 22, 30, 62], p38 kinase [6, 29], NFκB [35], calreticulin [63], caspase 12 [63] and GADD153 [63], inhibition of mitochondria permeability transition [34], cytochromes P450 1A1 and 1A2 [64], CYP3A2 and CYP2C11 [65], nitric oxide [5, 36], prostaglandin E2, CD40 [66] and matrix metalloproteinase-2 (MMP-2) activity [66]. At least in theory, some of those mechanisms may contribute to the stimulation of eryptosis. In any case, most of the effect of tanshinone IIA on eryptosis is explained by Ca^{2+} entry, ceramide formation and ATP depletion.

Tanshinone IIA sensitivity may be enhanced in clinical disorders associated with increased eryptosis-susceptibility of erythrocytes [37], such as iron deficiency [67], phosphate depletion [68], Hemolytic Uremic Syndrome [69], sepsis [70], sickle cell disease [71], malaria [72-76], APC gene mutation [77] Wilson's disease [76] and possibly metabolic syndrome [78]. Tanshinone IIA may further potentiate the eryptotic effect of other eryptosis triggering xenobiotics [48, 72, 79-92]. Accelerated eryptosis may lead to anemia [37] and adherence of phosphatidylserine-exposing erythrocytes to the vascular wall with the respective impairment of microcirculation [93-97]. Eryptotic erythrocytes are further known to stimulate blood clotting [93, 98, 99].

The present observations may be relevant not only for erythrocytes but similarly effective in nucleated cells. To the best of our knowledge, an effect of tanshinone on ceramide formation has never been reported, but possibly, ceramide similarly participates in the effects of tanshinone IIA on apoptosis, as ceramide is well known to trigger apoptosis in a variety of cells [100]. In nucleated cells, tanshinone influences apoptosis in part by inhibition of mitochondria permeability transition [34], a mechanism, which cannot be operative in erythrocytes. In nucleated cells, tanshinone IIA is further partially effective through inhibition of the transcription factor NFκB [35]. Again, transcription cannot contribute to suicidal death of mature, nuclei lacking erythrocytes. Interestingly, though, NFκB inhibitors Bay 11-7082 and Parthenolide similarly trigger eryptosis [85], an effect which may, however, not be related to NFκB inhibition.

In conclusion, the present study reveals a completely novel effect of tanshinone IIA, i.e. the Ca^{2+} entry, ATP depletion despite enhanced glycolysis, and ceramide formation in erythrocytes, which collectively stimulate cell membrane scrambling and cell shrinkage followed by clearance of the affected erythrocytes from circulating blood.

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