

Original Paper

Stimulation of Eryptosis, the Suicidal Erythrocyte Death by Piceatannol

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Ceramide • Calcium • Oxidative stress

Abstract

Background/Aims: Piceatannol, an analog and metabolite of resveratrol, is effective against various disorders including malignancy. It is in part effective by triggering suicidal death or apoptosis of tumor cells. Cellular mechanisms mediating the proapoptotic effect of Piceatannol include mitochondrial depolarization and cytochrome c release. Erythrocytes lack mitochondria but may nevertheless enter suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Cellular mechanisms involved in the triggering of eryptosis include increase of cytosolic Ca²⁺ activity ([Ca²⁺]_i), oxidative stress and ceramide formation. The present study explored, whether Piceatannol induces eryptosis and, if so, to shed some light on the cellular mechanisms involved. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, [Ca²⁺]_i from Fluo3-fluorescence, reactive oxygen species (ROS) formation from 2',7'-dichlorodihydrofluorescein (DCF) diacetate-dependent fluorescence, and ceramide abundance utilizing specific antibodies. Hemoglobin concentration in the supernatant was taken as measure of hemolysis. **Results:** A 48 hours exposure of human erythrocytes to Piceatannol (10 - 20 μM) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly increased DCFDA-fluorescence, significantly increased ceramide abundance, but did not significantly increase Fluo3-fluorescence. Removal of extracellular Ca²⁺ slightly blunted but did not abolish the effect of Piceatannol on annexin-V-binding and forward scatter. Piceatannol (20 μM) significantly augmented the increase of annexin-V-binding, but significantly blunted the decrease of forward scatter following treatment with the Ca²⁺ ionophore ionomycin. **Conclusions:** Piceatannol triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part downstream of Ca²⁺ and involving oxidative stress and ceramide formation.

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Introduction

Piceatannol (3,3',4,5'-tetrahydroxy-trans-stilbene), a naturally occurring hydroxylated analogue of resveratrol found in diverse plants including grapes and passion fruit [1-3], is effective against diverse disorders including hypercholesterolemia, arrhythmia, atherosclerosis and malignancy [1-3]. Piceatannol fosters vasodilation, counteracts angiogenesis as well as oxidative stress [2] and displays anti-inflammatory as well as antimicrobial activities [1]. Cellular mechanisms triggered by piceatannol include inhibition of cyclooxygenase activity [2], cell-cycle arrest [1]; upregulation of Bid, Bax, Bik, Bok, Fas as well as P21(WAF1) [1], down-regulation of Bcl-xL as well as BCL-2 [1], mitochondrial depolarization [1], cytochrome c release [1], and caspase activation [1]. Piceatannol modifies gene expression by downregulation of transcription factor NF- κ B [1] and Janus kinase JAK1 [1]. Depending on the cell type piceatannol may either stimulate [1, 4] or inhibit [1] apoptosis.

In analogy to apoptosis of nucleated cells erythrocytes may enter eryptosis [5], the suicidal erythrocyte death characterized by cell shrinkage [6] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [5]. Stimulators of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [5], ceramide [7], oxidative stress [5], energy depletion [5], activated caspases [5, 8, 9], stimulated activity of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [5], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [5]. Eryptosis could be stimulated by a wide variety of xenobiotics [5, 10-51].

In order to test whether eryptosis could be modified by Piceatannol, human erythrocytes drawn from healthy volunteers were exposed to Piceatannol and phosphatidylserine surface abundance, cell volume, $[Ca^{2+}]_i$, ROS formation, 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 hours. Where indicated, erythrocytes were exposed for 48 hours to Piceatannol (MedChem Express, Princeton, USA). In order to estimate the impact of Piceatannol on eryptosis due to high $[Ca^{2+}]_i$, erythrocytes were exposed for 30 min to a combination of Piceatannol and the Ca^{2+} ionophore ionomycin (Merck Millipore, Darmstadt, Germany).

FACS Analysis of Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 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). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and Piceatannol treated erythrocytes.

Measurement of Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min. 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.

Quantification of Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. After incubation, a 100 μ l suspension of erythrocytes was washed in Ringer solution and stained with DCF diacetate (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μ M. Erythrocytes were incubated at 37°C for 30 min in the dark and washed two times in Ringer solution. 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).

Determination of Ceramide formation

To determine ceramide abundance, a monoclonal antibody-based assay was used. To this end, 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. 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 analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As a control, secondary antibody alone was used.

Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Statistics

Data are expressed as arithmetic means \pm 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 Piceatannol stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. The measurements were performed after incubation of the erythrocytes 48 hours in Ringer solution without or with Piceatannol (5 – 20 μ M). As shown in Fig. 1A, B, Piceatannol slightly decreased the average erythrocyte forward scatter, an effect reaching statistical significance at 20 μ M Piceatannol. Fig. 1C, D demonstrates that Piceatannol significantly increased the percentage of both severely swollen and severely shrunken erythrocytes.

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Piceatannol (5 – 20 μ M). As shown in Fig. 2, a 48 hours exposure to Piceatannol increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 μ M Piceatannol. For comparison, the percentage of hemolysis after 48 hours exposure in Ringer solution without or with Piceatannol (5 – 20 μ M) is shown in the same bar chart (Fig. 2B; grey bars).

Fluo3-fluorescence was taken as a measure of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As a result, the average Fluo3-fluorescence was similar following a 48 hours incubation without Piceatannol as in the presence of Piceatannol (5 - 20 μ M) (Table 1).

Fig. 1. Effect of Piceatannol on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 μM Piceatannol. B. Arithmetic means \pm SEM (n = 10) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Piceatannol (5 - 20 μM). C. Arithmetic means \pm SEM (n = 10) of the percentage erythrocytes with forward scatter (FSC) <200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Piceatannol (5 - 20 μM). D. Arithmetic means \pm SEM (n = 10) of the percentage erythrocytes with forward scatter (FSC) >800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Piceatannol (5 - 20 μM). ** (p<0.01), *** (p<0.001) indicates significant difference from the absence of Piceatannol (ANOVA).

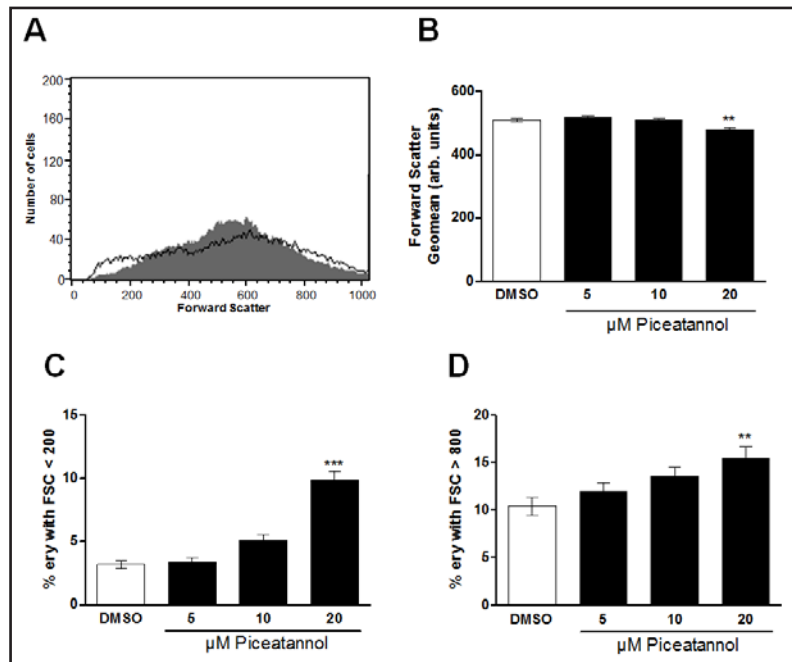


Fig. 2. Effect of Piceatannol on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 μM Piceatannol. B. Arithmetic means \pm SEM (n = 10) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Piceatannol (5 - 20 μM). For comparison, arithmetic means \pm SEM (n = 10) of the percentage of hemolysis is shown as grey bars. *** (p<0.001) indicates significant difference from the absence of Piceatannol (ANOVA).

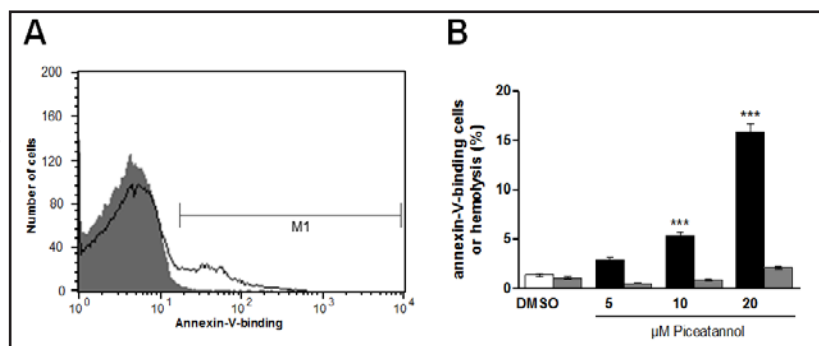


Table 1. Fluo3-fluorescence following incubation for 48 hours without or with Piceatannol treatment

	DMSO	5 μM	10 μM	20 μM
Fluo3-fluorescence	22.5 \pm 0.9 a.u., n=10	20.3 \pm 0.6 a.u., n=10	21.0 \pm 0.5 a.u., n=10	20.1 \pm 0.5 a.u., n=10

A next series of experiments explored whether the Piceatannol-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} . To this end, erythrocytes were incubated for 48 hours in the absence or presence of 20 μM Piceatannol in the presence or nominal absence of extracellular Ca^{2+} . As illustrated in Fig. 3, removal of extracellular Ca^{2+} slightly, but significantly blunted the effect of Piceatannol on

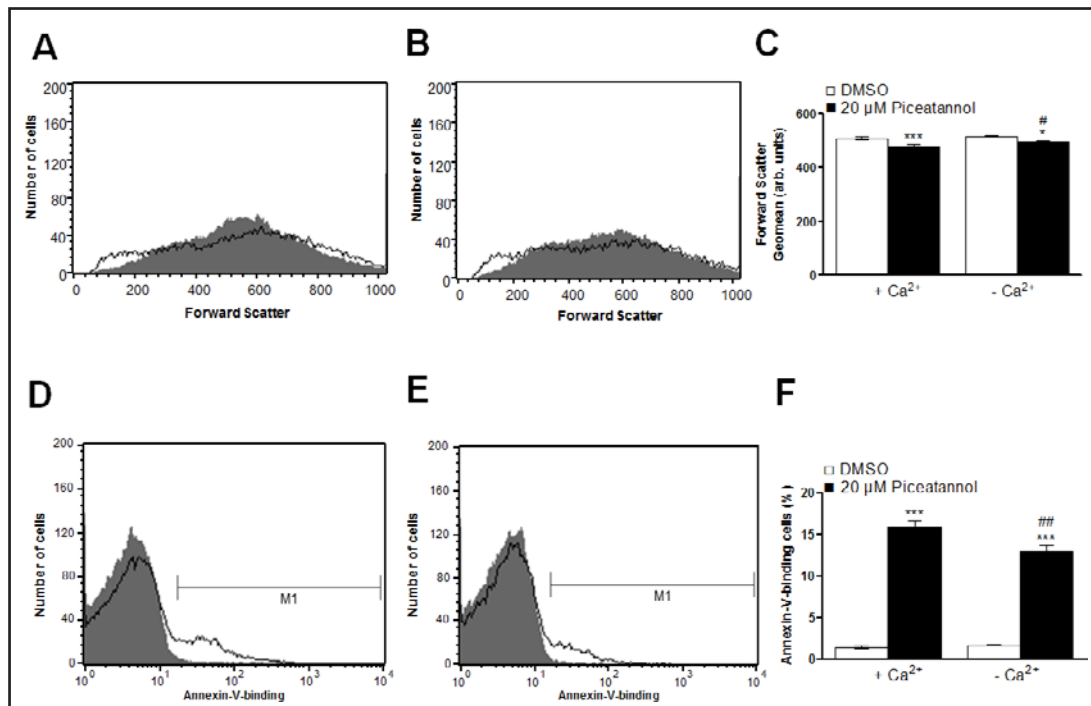


Fig. 3. Ca^{2+} sensitivity of Piceatannol-induced erythrocyte shrinkage and phosphatidylserine exposure. A,B. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Piceatannol (20 μ M) in the presence (A) and absence (B) of extracellular Ca^{2+} . C. Arithmetic means \pm SEM (n = 10) of forward scatter of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Piceatannol (20 μ M) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+} . D,E. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) Piceatannol (20 μ M) in the presence (D) and absence (F) of extracellular Ca^{2+} . F. Arithmetic means \pm SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Piceatannol (20 μ M) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+} . *(p<0.05), *** (p<0.001) indicates significant difference from the absence of Piceatannol, # (p<0.05), ## (p<0.01) indicates significant difference from the nominal absence of Ca^{2+} (ANOVA).

forward scatter. However, even in the absence of extracellular Ca^{2+} , Piceatannol significantly decreased the erythrocyte forward scatter. Similar observations were made with annexin-V-binding. Removal of extracellular Ca^{2+} slightly, but significantly blunted the effect of Piceatannol on annexin-V-binding. However, even in the absence of extracellular Ca^{2+} , Piceatannol significantly increased the percentage of annexin-V-binding erythrocytes (Fig. 3). Thus, Piceatannol-induced cell membrane scrambling was in large part triggered by mechanisms insensitive to entry of extracellular Ca^{2+} .

A next series of experiments explored whether Piceatannol modified cell shrinkage and translocation of phosphatidylserine following increase of cytosolic Ca^{2+} activity by treatment of the erythrocytes with Ca^{2+} ionophore ionomycin (1 μ M). To this end, erythrocytes were incubated for 48 hours in the absence or presence of 20 μ M Piceatannol and subsequently treated for 30 minutes with ionomycin (1 μ M). As illustrated in Fig. 4A following Piceatannol pretreatment, ionomycin increased cytosolic Ca^{2+} activity to similar values in erythrocytes with or without Piceatannol treatment. However, the effect of ionomycin on forward scatter was significantly blunted (Fig. 4C) and the effect of ionomycin on annexin-V-binding significantly stronger following Piceatannol pretreatment (Fig. 4B).

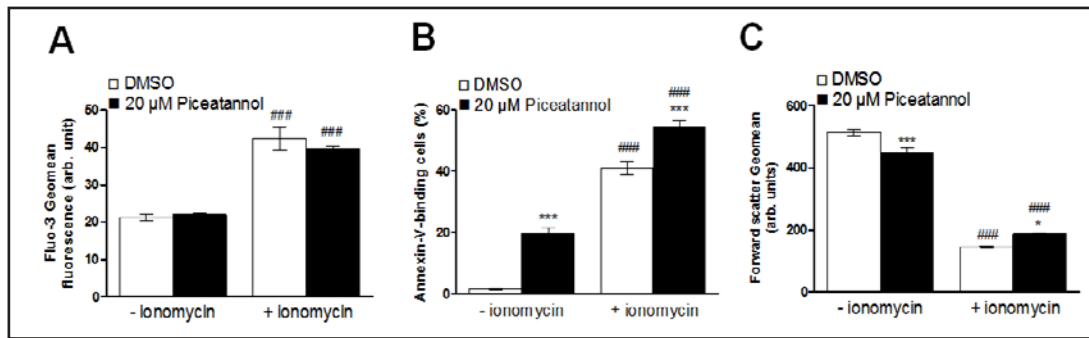


Fig. 4. Effect of Ca^{2+} ionophore ionomycin on phosphatidylserine exposure in the presence and absence of Piceatannol. A-C. Arithmetic means \pm SEM ($n = 10$) of (A) the Fluo3-fluorescence, (B) the percentage of annexin-V-binding erythrocytes, and (C) the forward scatter following incubation for 30 min in the absence (left bars, -ionomycin) or presence (right bars, +ionomycin) of Ca^{2+} ionophore ionomycin ($1 \mu\text{M}$) after a 48 hours preincubation in the absence (white bars) or presence (black bars) of $20 \mu\text{M}$ Piceatannol. *($p < 0.05$), ***($p < 0.001$) indicates significant difference from the absence of Piceatannol. ###($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

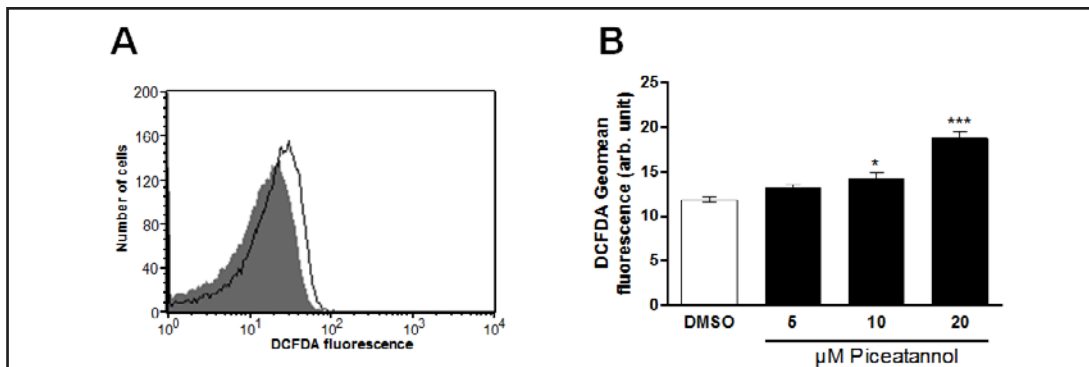


Fig. 5. Effect of Piceatannol on erythrocyte ROS formation. A. Original histogram of DCFDA-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of Piceatannol ($20 \mu\text{M}$). B. Arithmetic means \pm SEM ($n = 10$) of the DCFDA-fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) Piceatannol (5 – $20 \mu\text{M}$). *($p < 0.05$), ***($p < 0.001$) indicate significant difference from the absence of Piceatannol (ANOVA).

Eryptosis is further stimulated by oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing $2',7'$ -dichlorodihydrofluorescein (DCF) diacetate. As indicated in Fig. 5, the DCFDA-fluorescence was higher following exposure to Piceatannol than in the absence of Piceatannol, a difference reaching statistical significance at $10 \mu\text{M}$ Piceatannol concentration.

A further stimulator of eryptosis is ceramide. Ceramide abundance at the erythrocyte surface was thus quantified utilizing specific antibodies. As shown in Fig. 6, the ceramide abundance was significantly higher following exposure to $20 \mu\text{M}$ Piceatannol than in the absence of Piceatannol.

Discussion

The present observations reveal that exposure of human erythrocytes drawn from healthy individuals to the $3,3',4,5'$ -tetrahydroxy-trans-stilbene (Piceatannol) is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Thus, Piceatannol stimulates the suicidal erythrocyte death or eryptosis.

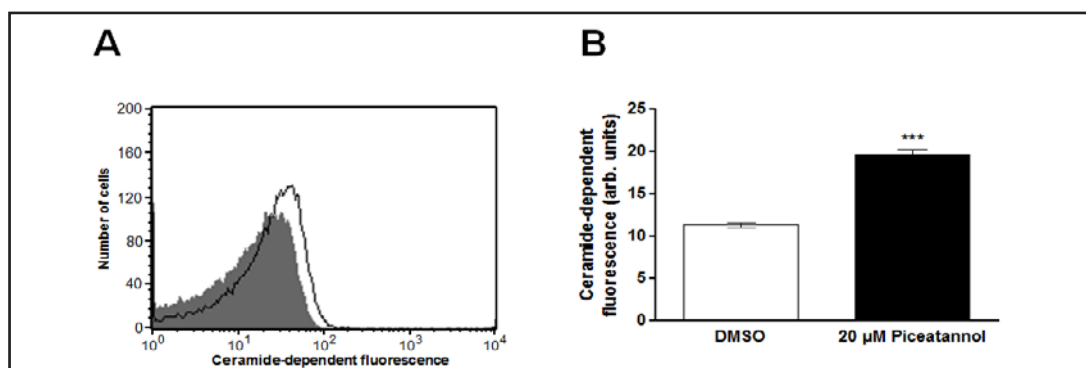


Fig. 6. Effect of Piceatannol on ceramide abundance at the erythrocyte surface. A. Original histogram of ceramide abundance in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 20 μM Piceatannol. B. Arithmetic means ± SEM (n = 10) of the ceramide abundance (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 20 μM Piceatannol. *** (p < 0.001) indicates significant difference from the absence of Piceatannol (ANOVA).

The experiments further shed some light on the cellular mechanisms involved. Piceatannol did not appreciably increase cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Instead, it slightly tended to decrease Fluo-3AM fluorescence, an effect, however, not reaching statistical significance. We cannot rule out an artifact, such as cellular loss of fluorescent dye. Nevertheless, the effect of Piceatannol on cell membrane scrambling was slightly, but significantly blunted following removal of extracellular Ca^{2+} . However, Piceatannol increased the percentage of annexin-V-binding erythrocytes even in the absence of extracellular Ca^{2+} . Moreover, Piceatannol augmented cell membrane scrambling in erythrocytes loaded with Ca^{2+} by treatment with the Ca^{2+} ionophore ionomycin. Thus, Piceatannol apparently sensitized the cells to the scrambling effect of Ca^{2+} and was effective downstream of Ca^{2+} . The sensitivity of cell membrane scrambling to Ca^{2+} could be enhanced by ceramide [5]. Piceatannol indeed enhanced the ceramide abundance, which could well contribute to or even account for the sensitization of cell membrane scrambling to $[\text{Ca}^{2+}]_i$. Moreover, the stimulation of cell membrane scrambling by Piceatannol was paralleled by oxidative stress, a major stimulator of eryptosis [5].

The effect of Piceatannol on cell shrinkage was again slightly, but significantly blunted by removal of Ca^{2+} from the extracellular space. On the other hand, Piceatannol significantly blunted the effect of the Ca^{2+} ionophore ionomycin on cell shrinkage. Ionomycin triggers cell shrinkage by increase of $[\text{Ca}^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water. Possibly, preincubation to Piceatannol was followed by impairment of Na^+/K^+ ATPase with gain of cytosolic Na^+ and loss of cellular K^+ . As a result, the K^+ exit, hyperpolarisation and Cl^- loss following opening of Ca^{2+} sensitive K^+ channels would be blunted by prior treatment with Piceatannol.

At low concentrations Piceatannol decreases and at high concentrations Piceatannol increases hemolysis and thus release of hemoglobin. Hemoglobin released into circulating blood may pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus lead to renal failure [52]. An important function of eryptosis is the disposal of defective erythrocytes prior to hemolysis. Eryptosis further accomplishes elimination of erythrocytes infected with the malaria pathogen *Plasmodium* [5].

Excessive eryptosis may, however, result in anemia, if the loss of eryptotic erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [5]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [53], stimulate blood clotting and trigger thrombosis [54-56], thus impairing microcirculation [7, 54, 57-60]. Enhanced eryptosis may thus contribute to the anemia and/or deranged microcirculation in several clinical conditions, such as chronic dehydration [61], hyperphosphatemia [62], chronic kidney disease (CKD) [63-66], hemolytic-uremic syndrome [67], diabetes [68], hepatic

failure [69], malignancy [5], sepsis [70], sickle-cell disease [5], beta-thalassemia [5], Hb-C and G6PD-deficiency [5], as well as Wilsons disease [71]. At least in theory, treatment of affected patients with Piceatannol may further aggravate anemia.

The effect of Piceatannol contrasts that of the related substance resveratrol [72-74], which has been shown to inhibit eryptosis [75].

In conclusion, Piceatannol triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently independent from Ca^{2+} entry, but involving oxidative stress and ceramide.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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