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Bromfenvinphos induced suicidal death of human erythrocytes

Arbace Officioso [a,b], Caterina Manna [b], Kousi Alzoubi [a], Florian Lang [a,*]

[a] Department of Physiology, University of Tübingen, Gmelinstr. 5, 72076 Tuebingen, Germany
[b] Department of Biochemistry, Biophysics and General Pathology, School of Medicine and Surgery, Second University of Naples, Via L. De Crecchio 7, 80138 Naples, Italy

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ABSTRACT

The organophosphorus pesticide bromfenvinphos ((E,Z)-O,O-diethyl-O-[1-(2,4-dichlorophenyl)-2-bromovinyl] phosphate) has been shown to decrease hematocrit and hemoglobin levels in blood presumably by triggering oxidative stress of erythrocytes. Oxidative stress is known to activate erythrocytic Ca^{2+} permeable unselective cation channels leading to Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), which in turn triggers eryptosis, the suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The present study explored, whether and how bromfenvinphos induces eryptosis. To this end, phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[Ca^{2+}]_i$ from Fluo3-fluorescence, and ROS formation from DCFDA dependent fluorescence. As a result, a 48 hour exposure of human erythrocytes to bromfenvinphos (≥100 μM) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly increased Fluo3-fluorescence, and significantly increased DCFDA fluorescence. The effect of bromfenvinphos on annexin-V-binding and forward scatter was significantly blunted, but not abolished by removal of extracellular Ca^{2+}. In conclusion, bromfenvinphos triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of ROS formation and Ca^{2+} entry.

1. Introduction

Bromfenvinphos ((E,Z)-O,O-diethyl-O-[1-(2,4-dichlorophenyl)-2-bromovinyl] phosphate), an organophosphorus pesticide with acaricidal and insecticidal potency, is widely used in agriculture [1,2]. In yeast, bromfenvinphos interferes with transport, respiration, fermentation and cell growth [3]. Bromfenvinphos inhibits acetylcholinesterase [2,4] and is toxic to the brain [5], liver [6], and kidney [6]. Following administration to mice, bromfenvinphos decreases hematocrit [7], blood hemoglobin level [8], and erythrocyte diameter [8]. The substance induces oxidative stress in human erythrocytes [1] without inducing hemolysis or hemoglobin oxidation [2].

Oxidative stress is a well known trigger of eryptosis [9], the suicidal death of erythrocytes characterized by cell shrinkage [10] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [9]. Oxidative stress opens Ca^{2+} permeable unselective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), which in turn stimulates cell membrane scrambling [9]. Signaling leading to eryptosis further includes ceramide [11], energy depletion [9], activated caspases [9,12,13], stimulated activity of casein kinase 1α [14], Janus-activated kinase JAK3 [15], protein kinase C [16], and p38 kinase [17], as well as impaired activity of AMP activated kinase AMPK [18], cGMP-dependent protein kinase [19], PAK2 kinase [20], and sorafenib/sunitinib sensitive kinases [9,21,22]. Eryptosis is stimulated by a myriad of xenobiotics [9,23–51].

The present study tested a putative effect of bromfenvinphos on eryptosis. To this end, human erythrocytes from healthy volunteers were treated with bromfenvinphos and phosphatidylserine surface abundance, cell volume as well as $[Ca^{2+}]_i$ and ROS formation determined by flow cytometry.

2. Materials and methods

2.1. 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 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 $CaCl_2$, at 37 °C for 24 h. Where indicated, erythrocytes were exposed to bromfenvinphos (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

* Corresponding author at: Physiologisches Institut der Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen, Germany.
E-mail address: florian.lang@uni-tuebingen.de (F. Lang).

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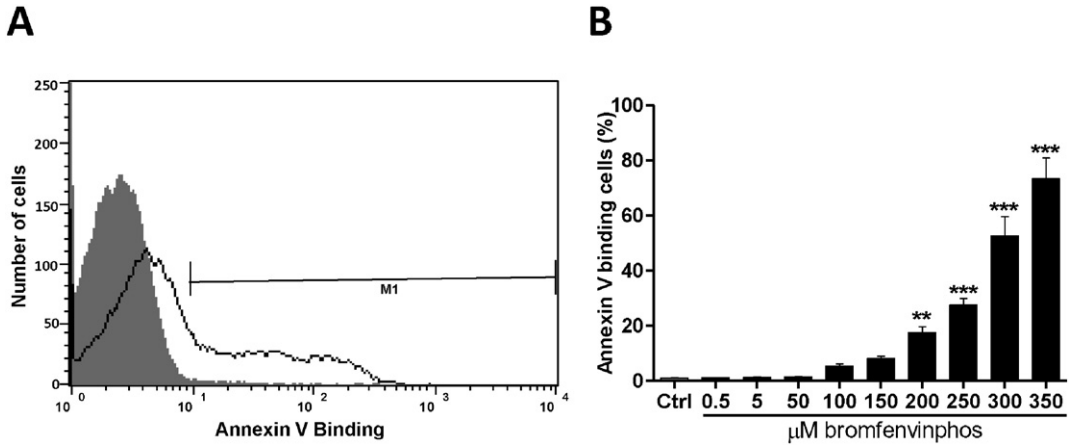

Fig. 1. Effect of bromfenvinphos on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 250 μM bromfenvinphos. B. Arithmetic means ± SEM (n = 4) of erythrocyte annexin-V-binding (black bars) following incubation for 48 h to Ringer solution without (Ctrl) or with presence of bromfenvinphos (0.5–250 μM). *($p < 0.05$),***($p < 0.001$) indicate significant difference from the absence of bromfenvinphos (ANOVA).

2.2. Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 150 μl cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37 °C for 20 min under protection from light. 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".

2.3. Hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 1600 rpm, room temperature) after incubation under the respective experimental conditions 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.

2.4. Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 5 μM Fluo-3/AM. The cells were incubated at 37 °C for 30 min and washed once in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

2.5. Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 μl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnelldorf, Germany) in PBS containing DCFDA at a final concentration of 10 μM. 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 μl 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).

2.6. Ceramide abundance at the erythrocyte surface

To determine ceramide abundance, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37 °C with 1 μg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing

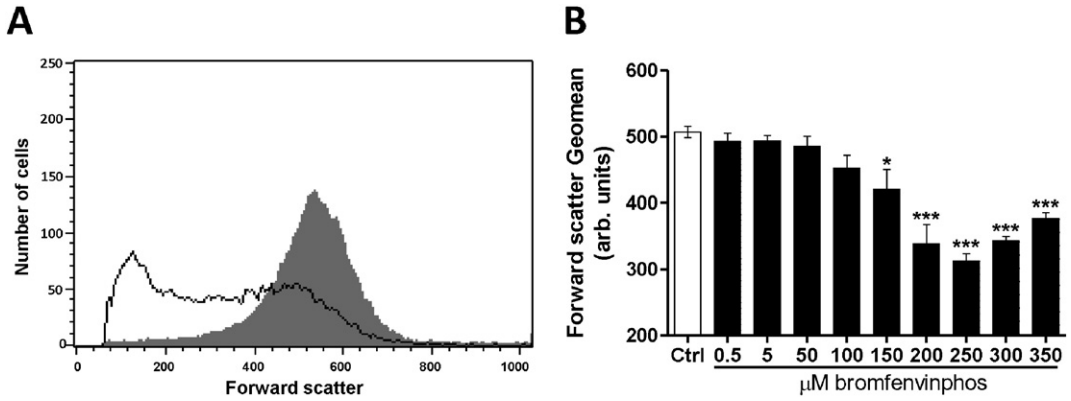

Fig. 2. Effect of bromfenvinphos on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (gray area) and with (black line) presence of 250 μM bromfenvinphos. B. Arithmetic means ± SEM (n = 4) of the erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) bromfenvinphos (0.5–250 μM). **($p < 0.01$),***($p < 0.001$) indicate significant difference from the absence of bromfenvinphos (ANOVA).