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Original Paper

Fucoxanthin Induced Suicidal Death of Human Erythrocytes

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract
Background/Aims: Fucoxanthin, a carotenoid isolated from brown seaweeds, induces suicidal death or apoptosis of tumor cells and is thus considered for the treatment or prevention of malignancy. In analogy to apoptosis of nucleated cell, erythrocytes may enter eryptosis, the suicidal death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress and activation of p38 kinase or protein kinase C. The present study explored, whether and how fucoxanthin induces eryptosis. ***Methods:*** 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 abundance of reactive oxygen species (ROS) from DCFDA dependent fluorescence and lipid peroxidation using BODIPY fluoresence. ***Results:*** A 48 hours exposure of human erythrocytes to fucoxanthin significantly increased the percentage of annexin-V-binding cells (≥ 50 μM), significantly decreased average forward scatter (≥ 25 μM), significantly increased hemolysis (≥ 25 μM), significantly increased Fluo3-fluorescence (≥ 50 μM), significantly increased lipid peroxidation, but did not significantly modify DCFDA fluorescence. The effect of fucoxanthin on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+}, and was insensitive to p38 kinase inhibitor skepinone (2 μM) and to protein kinase C inhibitor calphostin (100 nM). ***Conclusion:*** Fucoxanthin triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part due to stimulation of Ca^{2+} entry.

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Introduction

Fucoxanthin, a carotenoid from the chloroplasts of brown seaweeds [1-3] and/or its metabolite fucoxanthinol [1] have anti-mutagenic [1], anti-obesity [1-8], anti-diabetic [1, 3, 5, 6, 8], antioxidant [3, 4, 7, 8], anti-inflammatory [1, 4, 8], liver , skin, bone & eye protective [3, 8], cardio- & cerebrovascular protective [3, 4, 8], anti-angiogenic [8], and antimalarial [8] activities.

Fucoxanthinol and to a lesser extent fucoxanthin triggers apoptosis [1, 9-29] and is thus considered for the treatment or prevention of malignancy [1, 4, 7, 8, 15-17, 21, 22, 25, 28-31]. Signaling involved in the effect of fucoxanthin(ol) include NF-κB [1, 21, 24-27, 30], p53 [10], caspases [1], Bcl-2 proteins [1, 30], MAPK [1, 30], PI3K/Akt [1, 23, 26, 27], JAK/STAT [1, 28], AP-1 [1], GADD45 [1, 30], increase of cytosolic Ca^{2+} activity [20], and caspases [10, 12, 15-19, 23, 25, 29, 30]. The substance is further partially effective by stimulating autophagy [14] and by depolarizing mitochondria [5, 6, 27]. On the other hand, partially due to its antioxidant activity, fucoxanthin may inhibit apoptosis [32, 33].

Even though lacking mitochondria and nuclei, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell shrinkage [34] and by phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface [35]. Eryptosis is stimulated by Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), oxidative stress [35], ceramide [36], energy depletion [35], caspases [35, 37, 38], stimulation of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [35], as well as pharmacological or genetic knockout of AMP activated kinase AMPK, cGMP-dependent protein kinase, and PAK2 kinase and sorafenib/sunitinib sensitive kinases [35]. Eryptosis is stimulated by a wide variety of xenobiotics [35, 39-63] and is enhanced in diverse clinical conditions, such as dehydration [52], hyperphosphatemia [62] chronic kidney disease (CKD) [44, 64-66], hemolytic-uremic syndrome [67], diabetes [68], hepatic failure [69], malignancy [35], sepsis [70], sickle-cell disease [35], beta-thalassemia [35], Hb-C and G6PD-deficiency [35], as well as Wilsons disease [71].

The present study explored whether and how fucoxanthin triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed for 48 hours to fucoxanthin and phosphatidylserine surface abundance, cell volume as well as $[Ca^{2+}]_i$ and ROS formation quantified 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 $MgSO_4$, 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 fucoxanthin (Sigma Aldrich, Hamburg, Germany), skepinone [72], or calphostin (Cayman, Ann Arbor, USA) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 100 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".

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Hemolysis

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 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.

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 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.

Reactive oxygen 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).

Lipid peroxidation

For detection of lipid peroxidation BODIPY 581/591 C11 (Thermo Fisher Scientific, MA, USA) was used. The nonoxidized dye has an emmission wavelength of 590 nm (FL2) and loses fluorescence to 510 nm (FL1) upon interaction with peroxyl radicals. Following the respective treatment, cells were loaded with 5 μM BODIPY for 30 min at 37°C in the dark. Cells were collected and analyzed by FACS Calibur (BD) at emission wavelengths of 530 nm and 585 nm.

Thiazole Orange

For studying the effect of fucoxanthin on the reticulocyte sub- population thiazole orange was used according to methods described previously [73]. Briefly, treated samples where stained with 1 μg/ml Thiazole orange for 30 min in the dark at 37°C and measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Analysis of the plot of forward scatter vs. fluorescence of the stained samples enables the discrimination of reticulocytes from the rest of erythrocyte population, thereafter gates where set on the two population and binding to Annexin-V- Alexa Fluor™ 647 (Thermo Fisher Scientific, MA, USA) and forward scatter was determined for each.

Statistics

Data are expressed as arithmetic means ± 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 the putative effect of fucoxanthin on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Annexin-V-binding was determined by flow cytometry in order to identify phosphatidylserine exposing erythrocytes. Prior to measurement, erythrocytes were incubated for 48 hours in Ringer solution without or with fucoxanthin (25 - 75 μM). As illustrated in Fig. 1, a 48 hours exposure to fucoxanthin

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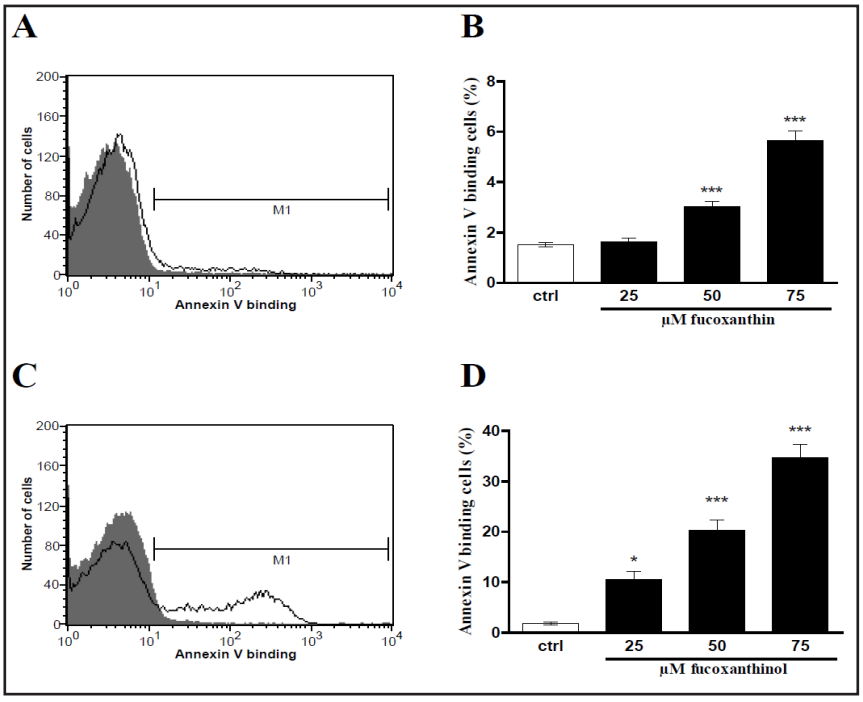

Fig. 1. Effect of fucoxanthin on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 µM fucoxanthin. B. Arithmetic means ± SEM (n = 14) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthin (25 - 75 µM). C. 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 75 µM fucoxanthinol. D. Arithmetic means ± SEM (n = 4) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthinol (25 - 75 µM). * (p<0.05), ***(p<0.001) indicates significant difference from the absence of fucoxanthin (ANOVA).

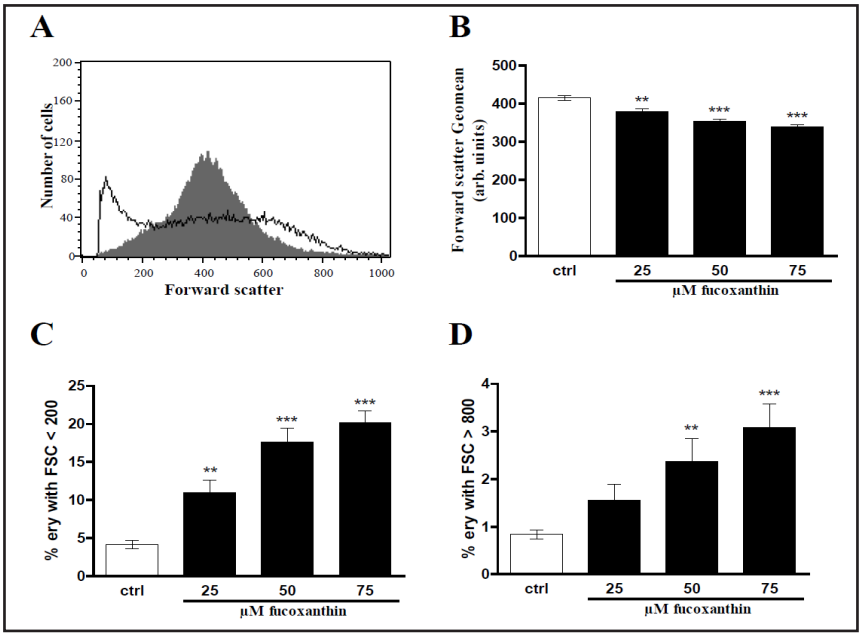

Fig. 2. Effect of fucoxanthin on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 75 µM fucoxanthin. B. Arithmetic means ± SEM (n = 14) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25 - 75 µM). C. Arithmetic means ± SEM (n = 14) of the percentage erythrocytes with forward scatter (FSC) < 200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25- 75 µM). D. Arithmetic means ± SEM (n = 14) of the percentage erythrocytes with forward scatter (FSC) > 800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25- 75 µM). **(p<0.01),***(p<0.001) indicate significant difference from the absence of fucoxanthin (ANOVA).

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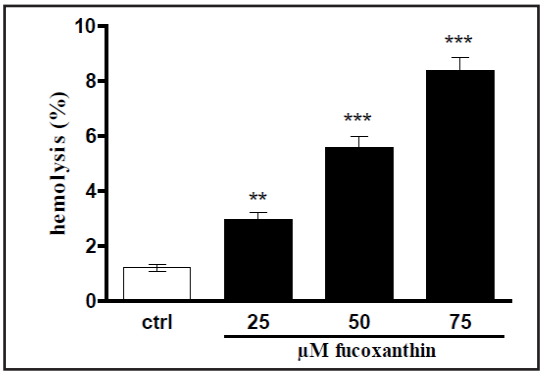

Fig. 3. Effect of fucoxanthin on hemolysis. Arithmetic means ± SEM (n = 10) of the percentage hemolysed erythrocytes following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of fucoxanthin (25 - 75 µM). **(p<0.01),***(p<0.001) indicates significant difference from the absence of fucoxanthin (ANOVA).

increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 50 µM fucoxanthin. Similarly, fucoxanthinol treatment for 48 hours increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 25 µM.

Cell volume was estimated by determination of forward scatter with flow cytometry. Prior to measurements the erythrocytes were again incubated for 48 hours in Ringer solution without or with fucoxanthin (25 – 75 µM). As shown in Fig, 2, fucoxanthin decreased the average erythrocyte forward scatter, an effect reaching statistical significance at 50 µM fucoxanthin concentration. Inspection of the histogram reveals, however, that a subpopulation of erythrocytes underwent cell shrinkage but that another subpopulation of erythrocytes underwent cell swelling. Accordingly, exposure of erythrocytes to fucoxanthin was followed by an increase of the percentage of both small (Fig. 2C) and large (Fig. 2D) erythrocytes, alterations reaching significance at fucoxanthin concentrations of 25 µM for small erythrocytes and 50µM for large erythrocytes. In order to explore whether fucoxanthin exerts opposing effects on reticulocytes and on mature erythrocytes, thiazole orange was utilized in order to identify reticulocytes. As a result, fucoxanthin increased the percentage reticulocytes binding annexin-V from 15.6 ± 1.8% to 25.1 ± 1.7% (n = 9) and the percentage of mature erythrocytes binding annexin-V from 6.7 ± 1.3% to 16.0 ± 0.9% (n = 9). Fucoxanthin decreased forward scatter in reticulocytes from 810 ± 46 to 634 ± 32 (n = 9) and in mature erythrocytes from 716 ± 19 to 320 ± 33.6 (n = 9). The respective values were not significantly different between reticulocytes and mature erythrocytes.

Further experiments addressed the putative effect of fucoxanthin on hemolysis. To this end, the hemoglobin concentration in the supernatant was determined by photometry. As illustrated in Fig. 3, a 48 hours incubation of erythrocytes in the presence of fucoxanthin was followed by a significant increase of the percentage of hemolytic erythrocytes, an effect reaching statistical significance at 25 µM fucoxanthin concentration.

Fluo3-fluorescence was quantified in order to estimate cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). As illustrated in Fig. 4, a 48 hours exposure to fucoxanthin increased the Fluo3-fluorescence, an effect reaching statistical significance at 50 µM fucoxanthin. In order to test whether fucoxanthin -induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+}, erythrocytes were incubated for 48 hours in the absence or presence of 75 µM fucoxanthin in the presence or nominal absence of extracellular Ca^{2+}. As shown in Fig 5, removal of extracellular Ca^{2+} significantly blunted the effect of fucoxanthin on annexin-V-binding. However, fucoxanthin significantly increased the percentage of annexin-V-binding erythrocytes even in the absence extracellular Ca^{2+}. Thus, fucoxanthin-induced cell membrane scrambling was partially but not completely due to entry of extracellular Ca^{2+}.

In order to quantify reactive oxygen species (ROS) abundance, 2′,7′-dichlorodihydro-fluorescein diacetate (DCFDA) fluorescence was determined utilizing flow cytometry. As a result, the ROS abundance was similar following a 48 hours exposure in the absence of fucoxanthin (18.2 ± 0.7 a.u., n = 10) and in the presence of 25 µM (16.5 ± 0.9 a.u., n = 10), 50 µM (16.3 ± 1.0 a.u., n = 10) and 75 µm (17.6 ± 0.9 a.u., n = 10) fucoxanthin. Thus, fucoxanthin did not induce appreciable oxidative stress.

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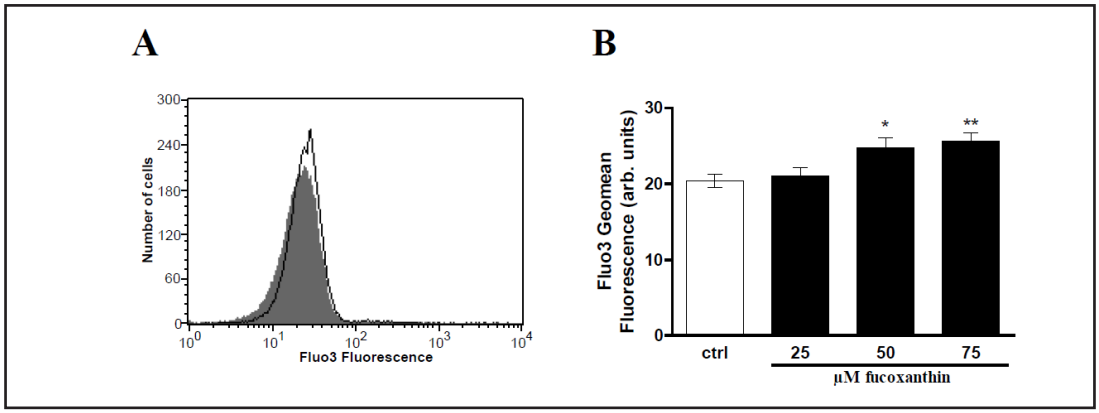

Fig. 4. Effect of fucoxanthin on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3-fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of fucoxanthin (75 µM). B. Arithmetic means ± SEM (n = 14) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) fucoxanthin (25 – 75 µM). *(p<0.05),***(p<0.01) indicate significant difference from the absence of fucoxanthin (ANOVA).

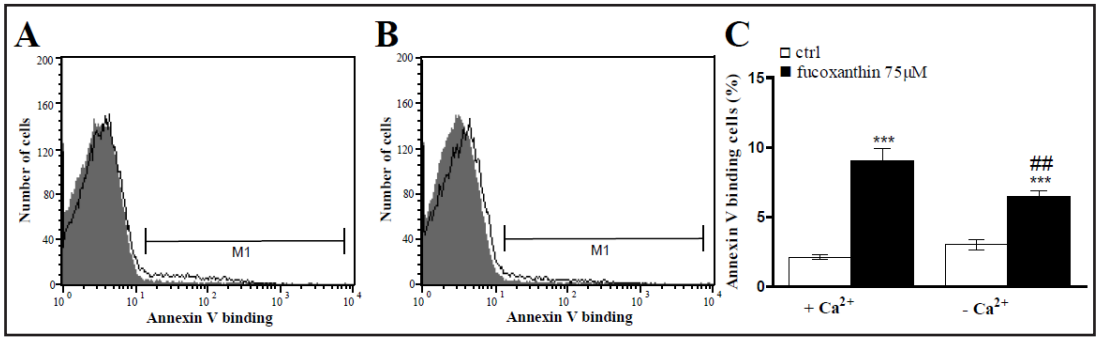

Fig. 5. Ca^{2+} sensitivity of fucoxanthin -induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of fucoxanthin (75 µM) in the presence (A) and absence (B) of extracellular Ca^{2+}.C. Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) fucoxanthin (75 µM) in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+}. ***(*P*<0.001) indicates significant difference from the absence of fucoxanthin, ##(p<0.01) indicate significant difference from the presence of Ca^{2+} (ANOVA).

In order to quantify lipid peroxidation, BODIPY 581/591 C11 fluorescence was determined in flow cytometry. As illustrated in Fig. 6, a 48 hours incubation of erythrocytes in the presence of fucoxanthin was followed by a significant increase of fluorescence of the oxidized form and significant decrease in the fluorescence of the nonoxidized form of BODIPY. Thus, fucoxanthin significantly increased lipid peroxidation.

To explore whether stimulation of annexin-V-binding by fucoxanthin required the activity of p38 kinase or protein kinase C, experiments were performed in the absence and presence of the p38 kinase inhibitor skepinone (2 µM) or of the protein kinase C inhibitor calphostin (100 nM). As a result, a 48 hours exposure to fucoxanthin (75 µM) increased the percentage of annexin-V-binding erythrocytes to a similar extent in the absence of the kinase inhibitors (from 2.0 ± 0.3 to 7.6 ± 0.2 %, n = 6), the presence of skepinone (from 2.1 ± 0.2 to 6.8 ± 0.3 %, n = 6), and in the presence of calphostin (from 2.1 ± 0.3 to 7.4 ± 0.5 %, n = 6). Thus p38 kinase or protein kinase C activities are apparently not required for the effect of fucoxanthin on erythrocyte cell membrane scrambling.

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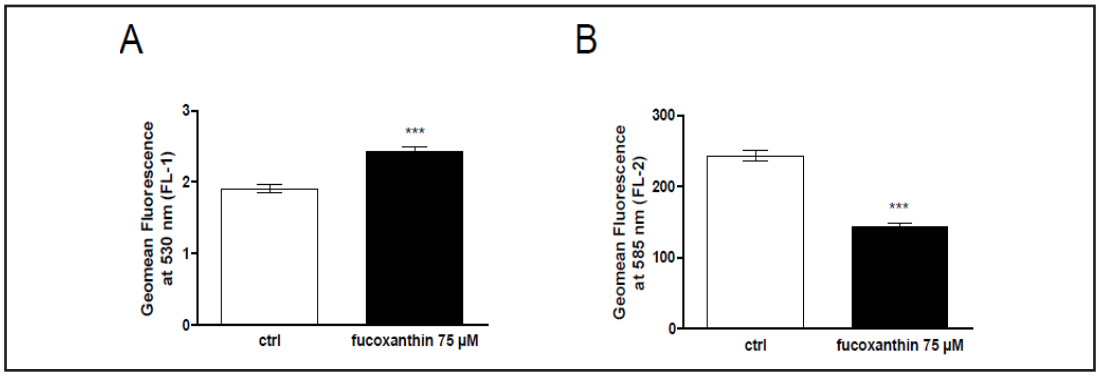

Fig. 6. Effect of fucoxanthin on lipid peroxidation. Arithmetic means ± SEM (n = 9) of geomean fluorescence at (A) 530 nm and (B) 585 nm emission wavelength in erythrocytes exposed for 48 hours to Ringer solution without (white bars) or with (black bars) fucoxanthin (75 µM). ***(P<0.001) indicates significant difference from the absence of fucoxanthin.

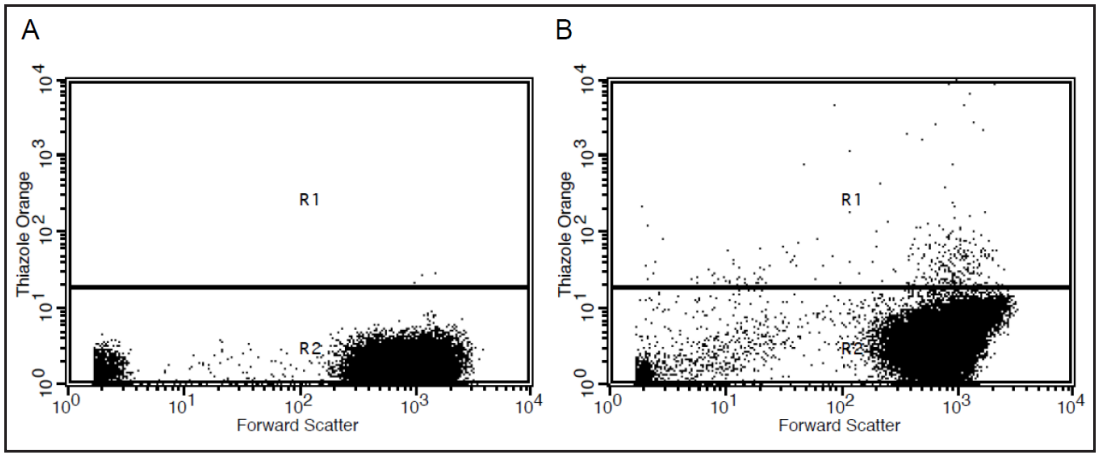

Fig. 7. Dot plots of thiazole orange staining. Original dot plots of forward scatter vs thiazole orange fluorescence in erythrocytes unstained (A) and Stained (B) with 1 µg/ml thiazole orange as prescribed in materials and methods, gates R1, R2 where set to determine reticulocytes from mature erythrocytes.

Discussion

The present observations disclose a novel effect of fucoxanthin, i.e. the triggering of eryptosis, the suicidal erythrocyte death. Treatment of human erythrocytes with fucoxanthin is followed by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface.

The effect of fucoxanthin on cell membrane scrambling was in part due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), a well-known stimulator of cell membrane scrambling [35]. Accordingly, removal of extracellular Ca^{2+} significantly blunted the stimulation of cell membrane scrambling by fucoxanthin. Ca^{2+} entered presumably through Ca^{2+} permeable cation channels. Stimulators of those channels include oxidative stress [35]. However, according to DCFDA fluorescence, fucoxanthin treatment did not appreciably increase the abundance of reactive oxygen species. Moreover, stimulation of erythrocyte cell membrane scrambling by fucoxanthin did not require activation of skepinone and calphostin sensitive kinases.

Beyond its effect on cell membrane scrambling, the increase of $[Ca^{2+}]_i$ following fucoxanthin treatment is expected to activate Ca^{2+} sensitive K^+ channels with subsequent cell shrinkage due to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [34]. Accordingly, the fucoxanthin treatment was followed by a significant decrease of the average forward scatter. However, close inspection of the histogram reveals that following fucoxanthin treatment a subpopulation of erythrocytes decreases, whereas another

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subpopulation of erythrocytes increases forward scatter. Thus, fucoxanthin treatment leads to swelling of a subset of erythrocytes.

The results with thiazole orange staining suggest that fucoxanthin increased the percentage of annexin-V-binding reticulocytes and mature erythrocytes. The relative effect was higher in mature erythrocytes, an observation possibly pointing to enhanced susceptibility of mature erythrocytes. Enhanced susceptibility of aged erythrocytes to oxidative stress and eryptosis has been shown previously [74].

Erythrocyte swelling may eventually result in hemolysis. Hemolysis is followed by release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules and thus occludes nephrons [75]. It is the purpose of eryptosis to clear defective erythrocytes from circulating blood prior to hemolysis [35]. Eryptosis further allows the clearance of erythrocytes infected with the malaria pathogen *Plasmodium.* In the infected host erythrocyte *Plasmodium* imposes oxidative stress, which activates the Ca^{2+}-permeable erythrocyte cation channels [35, 76]. The susceptibility of erythrocytes from carriers of sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency to eryptosis is enhanced and thus the clearance of infected erythrocytes accelerated. As a result parasitemia is decreased and the carriers of those blood disorders are partially protected against a severe course of malaria [35, 77-79]. A similar protection is provided by clinical conditions with accelerated eryptosis, such as iron deficiency [80], or by xenobiotics triggering eryptosis, such as lead [80], chlorpromazine [81] or NO synthase inhibitors [81]. If the additive effect of oxidative stress in infected erythrocytes imposed by the intracellular pathogen [35, 76] and the sensitizing effect of fucoxanthin to stimulators of eryptosis leads to the preferential eryptosis and clearance of infected erythrocytes, fucoxanthin treatment may be similarly beneficial for the clinical course of malaria.

However, excessisve eryptosis may lead to anemia as soon as it is not matched by a similar increase of erythropoiesis [35]. Moreover, stimulation of eryptosis may impair microcirculation [36, 82-86] by adherence of phosphatidylserine exposing erythrocytes to the vascular wall [87], stimulation of blood clotting and triggering of thrombosis [82, 88, 89].

Conclusion

Fucoxanthin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to increase of cytosolic Ca^{2+} activity.

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Disclosure Statement

The sponsor have had played no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

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