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Apigenin-Induced Suicidal Erythrocyte Death

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ABSTRACT: Apigenin, a flavone in fruits and vegetables, stimulates apoptosis and thus counteracts cancerogenesis. Erythrocytes may similarly undergo suicidal cell death or eryptosis, characterized by cell shrinkage and phosphatidylserine exposure at the cell surface. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), ceramide formation and ATP depletion. The present study explored the effect of apigenin on eryptosis. $[Ca^{2+}]_i$ was estimated from Fluo3-fluorescence, cell volume from forward scatter, phosphatidylserine exposure from annexin V binding, hemolysis from hemoglobin release, ceramide utilizing antibodies, and cytosolic ATP with luciferin−luciferase. A 48 h exposure to apigenin significantly increased $[Ca^{2+}]_i$ (≥1 μM), increased ceramide formation (15 μM), decreased ATP concentration (15 μM), decreased forward scatter (≥1 μM), and increased annexin V binding (≥5 μM) but did not significantly modify hemolysis. The effect of 15 μM apigenin on annexin V binding was blunted by Ca^{2+} removal. The present observations reveal novel effects of apigenin, i.e. stimulation of Ca^{2+} entry, ceramide formation and ATP depletion in erythrocytes with subsequent triggering of suicidal erythrocyte death, paralleled by cell shrinkage and phosphatidylserine exposure.

KEYWORDS: *phosphatidylserine, apigenin, calcium, cell volume, eryptosis*

INTRODUCTION

Apigenin, a flavone from fruits, vegetables and further plant materials,[1−19] counteracts inflammation, oxidative stress and development of cancer.[19−22] The protective effect of apigenin against tumor growth is at least partially due to induction of apoptosis.[23−27] On the other hand, apigenin may protect against apoptosis.[28] The apoptosis stimulating effect is at least partially due to influence of apigenin on mitochondria[26,29] and on gene expression.[30−33] Apigenin is partially effective through targeting of the JAK/STAT pathway.[32]

Even though erythrocytes are devoid of mitochondria and nuclei,[34] key organelles in suicidal death of nucleated cells, they may similarly undergo suicidal cell death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage.[34] Triggers of eryptosis include Ca^{2+} entry through Ca^{2+}-permeable cation channels.[35,36] Ca^{2+} activates Ca^{2+}-sensitive K^+ channels[37] with subsequent K^+ exit, hyperpolarization, Cl^- exit and thus cellular KCl loss with osmotically obliged water, resulting in cell shrinkage.[38] Ca^{2+} further triggers cell membrane scrambling with subsequent exposure of phosphatidylserine at the cell surface.[39] Triggers of eryptosis further include ceramide formation[40] and energy depletion.[41] Eryptosis is further triggered by inhibition of JAK3.[42]

The present study explored whether apigenin triggers eryptosis and whether the effect of apigenin involves alterations of cytosolic Ca^{2+} activity, cytosolic ATP levels and/or ceramide formation.

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 (184/2003 V).

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. Where indicated, erythrocytes were exposed to apigenin (Tocris, Bristol, U.K.) or γ-aminobutyric acid (Sigma, Schnelldorf, Germany) at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 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 conditions, 50 μL of 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. 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 erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, CA, 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 of 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 was incubated for 48 h at 37 °C in Ringer solution with or without apigenin and in Ringer solution with or without extracellular calcium (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution as a positive control. All subsequent manipulations were 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

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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 Ceramide Formation. For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without apigenin, 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 analyzed by flow cytometric analysis in FL-1.

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.

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 eryptotic effects, the same erythrocyte specimens have been used for control and experimental conditions.

RESULTS

In order to determine whether apigenin influences cytosolic Ca^{2+} concentration, Fluo 3 fluorescence was determined in FACS analysis. As illustrated in Figure 1, treatment of human

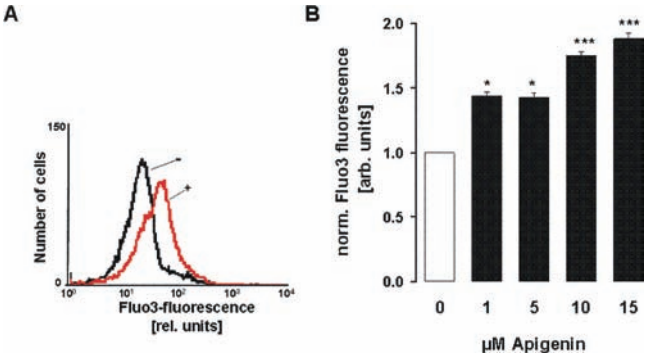

Figure 1. Effect of apigenin on erythrocyte cytosolic Ca^{2+} concentration. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) presence of 15 μM apigenin. (B) Arithmetic means ± SEM (n = 12−16) of the normalized 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) 1−15 μM apigenin. * (p < 0.05) and *** (p < 0.001) indicate significant difference from the absence of apigenin (ANOVA).

erythrocytes with apigenin resulted in an increase of Fluo3 fluorescence. The effect was statistically significant at all concentrations employed (≥1 μM). Thus, apigenin increases cytosolic Ca^{2+} concentration.

An increase of cytosolic Ca^{2+} concentration in erythrocytes is expected to activate Ca^{2+}-sensitive K^+ channels, which should trigger exit of KCl with osmotically obliged water and should thus result in cell shrinkage. Forward scatter in FACS analysis was thus taken as a measure of cell volume. As illustrated in Figure 2, apigenin treatment was indeed followed by a decrease

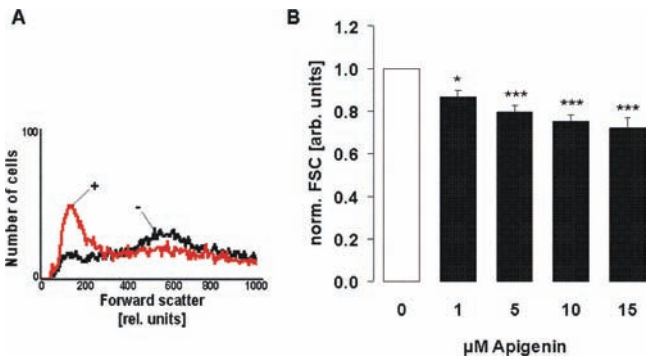

Figure 2. Effect of apigenin on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) presence of 15 μM apigenin. (B) Arithmetic means ± SEM (n = 12−16) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) 1−15 μM apigenin. * (p < 0.05) and *** (p < 0.001) indicate significant difference from the absence of apigenin (ANOVA).

of forward scatter, an effect again statistically significant at all concentrations employed (≥1 μM).

An increased cytosolic Ca^{2+} activity is further known to trigger cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface. Thus, annexin V binding was employed to identify phosphatidylserine exposing erythrocytes. Annexin V binding was quantified by FACS analysis. As illustrated in Figures 3A and 3B, a 48 h treatment with apigenin

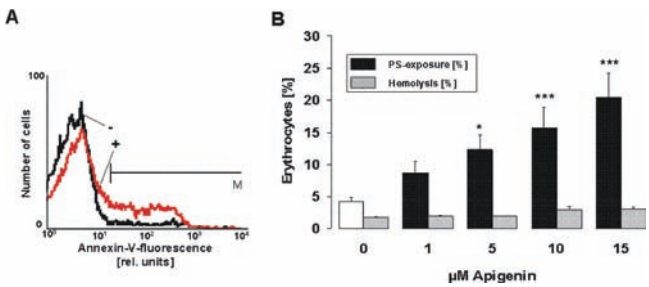

Figure 3. Effect of apigenin on phosphatidylserine exposure. (A) Original histogram of annexin V binding of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) 15 μM apigenin. (B) Arithmetic means ± SEM (n = 10) of erythrocyte annexin V binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) 1−15 μM apigenin. For comparison, arithmetic means ± SEM (n = 4) of the percentage of hemolysis are shown as gray bars. * (p < 0.05) and *** (p < 0.001) indicate significant difference from the absence of apigenin (ANOVA).

indeed increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at ≥5 μM apigenin.

In a further series of experiments hemolysis was estimated from hemoglobin release into the supernatant. As a result,

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exposure of erythrocytes for 48 h to Ringer solution with apigenin tended to increase hemoglobin concentration in the supernatant, an effect, however, not reaching statistical significance (Figure 3 B).

Further experiments addressed the role of Ca^{2+} entry in the triggering of erythrocyte cell membrane scrambling. To this end, erythrocytes were exposed to 15 μM apigenin in the presence or in the nominal absence of extracellular Ca^{2+}. As shown in Figure 4, the effect of apigenin on annexin V binding

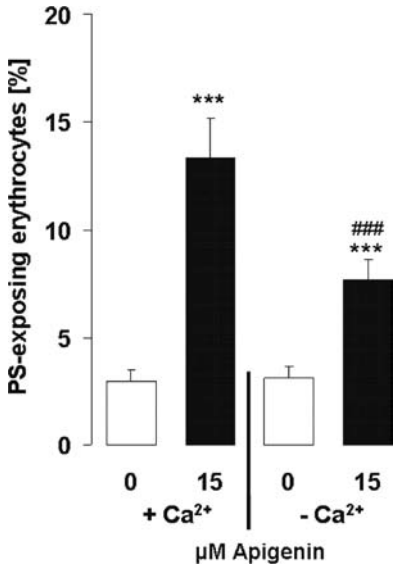

Figure 4. Effect of Ca^{2+} withdrawal on apigenin- induced annexin V binding. Arithmetic means ± SEM (n = 12) of the percentage of annexin V binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 15 μM apigenin in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of calcium. *** (p < 0.001) indicates significant difference from the absence of apigenin (ANOVA), and ### (p < 0.001) indicates significant difference from the respective values in the presence of Ca^{2+}.

was significantly blunted in the nominal absence of Ca^{2+}, an observation confirming that Ca^{2+} entry contributed to the triggering of erythrocyte cell membrane scrambling following apigenin exposure. However, even in the nominal absence of Ca^{2+} the percentage of annexin V binding erythrocytes was still significantly enhanced by apigenin exposure, an observation pointing to additional mechanisms contributing to the stimulation of erythrocyte membrane scrambling by apigenin. The effect of apigenin on cell membrane scrambling is thus only in part secondary to an increase of intracellular Ca^{2+} activity.

Additional experiments explored whether γ-aminobutyric acid (GABA) modified apigenin-induced phosphatidylserine exposure in erythrocytes. To this end, erythrocytes were incubated in the presence and absence of 15 μM apigenin and in the presence and absence of 15 μM GABA. In the absence of GABA, the percentage of annexin V binding erythrocytes increased significantly from 2.7 ± 0.5% (n = 6) in the absence of apigenin to 12.3 ± 1.8% (n = 6) in the presence of 15 μM apigenin. Addition of 15 μM GABA, did not significantly modify the percentage of annexin V binding erythrocytes in the absence of apigenin (3.5 ± 0.9%, n = 6) nor in the presence of 15 μM apigenin (12.1 ± 2.2%, n = 6).

Additional experiments were performed to investigate whether the PS-exposure induced by apigenin was reversible. To this end, PS-exposure was quantified using annexin V binding after 48 h. Erythrocytes incubated for 48 h in the presence of 15 μM apigenin (19.6 ± 2.3%, n = 4) induced significantly higher PS exposure in comparison to erythrocytes incubated in the absence of apigenin (3.2 ± 0.5%, n = 4). The samples were then aliquoted into two. The first aliquot was washed 4 times with Ringer solution and then incubated for an additional 24 h, while the second aliquot was not washed and was incubated for an additional 24 h. Following 72 h, the PS exposure in the washed aliquot (34.8 ± 2.9%, n = 4) did not significantly differ from the unwashed aliquot (32.9 ± 4.3%, n = 4) pointing to irreversibility of apigenin-induced phosphatidylserine exposure.

A further series of experiments thus explored the effect of apigenin treatment on the formation of ceramide, which would be similarly expected to trigger cell membrane scrambling. Ceramide formation was quantified utilizing FITC-labeled anti-ceramide antibodies. As illustrated in Figures 5A and 5B, the

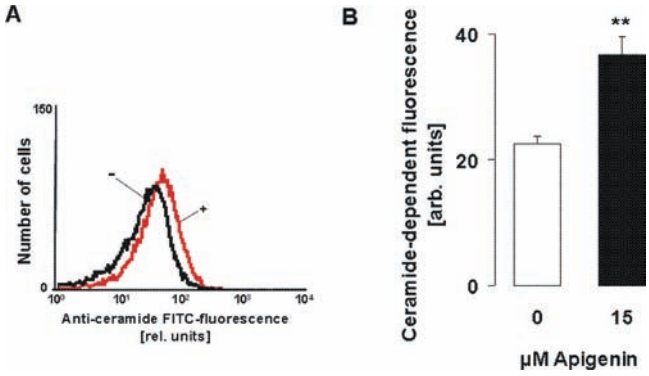

Figure 5. Effect of apigenin on ceramide formation. (A) Original histogram of anti-ceramide FITC fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, red line) 15 μM apigenin. (B) Arithmetic means ± SEM (n = 4) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) apigenin (15 μM). ** (p < 0.01) indicates significant difference from control (absence of apigenin) (t test).

ceramide-dependent fluorescence was significantly lower following a 48 h exposure to Ringer containing 15 μM apigenin than following exposure to Ringer solution without apigenin. Thus, apigenin indeed significantly enhanced ceramide formation.

A final series of experiments explored whether apigenin treatment influences cytosolic ATP concentration. As illustrated in Figure 6, the cytosolic ATP concentration in erythrocytes was significantly lower following a 48 h exposure to Ringer containing 15 μM apigenin than following exposure to Ringer solution without apigenin. Thus, apigenin indeed resulted in ATP depletion. Exposure to glucose-free Ringer served as a positive control. As expected, cytosolic ATP concentration was significantly decreased following a 48 h exposure to glucose depleted Ringer solution. Additional experiments explored whether the decrease in intracellular ATP induced by apigenin is Ca^{2+}-sensitive. As a result, erythrocytes incubated in Ca^{2+}-free Ringer solution and treated with apigenin (15 μM) for 48 h had significantly lower intracellular ATP content (0.2 ± 0.03 mM; n = 4) than erythrocytes incubated in

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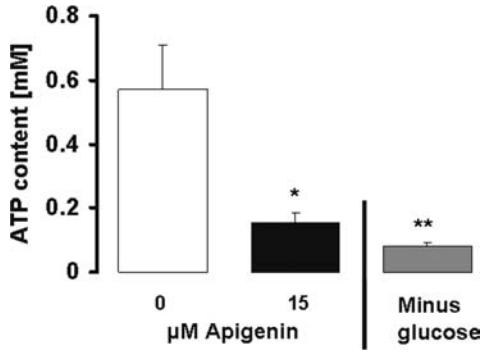

Figure 6. Effect of apigenin on erythrocyte cytosolic ATP content. Arithmetic means ± SEM (n = 4) of the ATP concentration after a 48 h incubation in Ringer solution without (white bar) or with (black bars) 15 μM apigenin, or in glucose-depleted Ringer solution (gray bar, minus glucose). * ($p < 0.05$) and ** ($p < 0.01$) indicate significant difference from control (absence of apigenin and presence of glucose) (ANOVA).

Ca^{2+}-free Ringer solution without apigenin (0.7 ± 0.1 mM; n = 4). Thus, apigenin decreased intracellular ATP content in the presence and absence of extracellular Ca^{2+}.

DISCUSSION

The present observation reveals a novel effect of apigenin, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Specifically, apigenin stimulates cell membrane scrambling and leads to cell shrinkage. The concentration required for the effect on cell membrane scrambling is well in the range of concentrations observed *in vivo*.[43]

Apigenin is partially effective by stimulating Ca^{2+} entry with subsequent increase of cytosolic Ca^{2+} activity. Apigenin presumably activates cation nonselective channels, which have previously been shown to somehow involve TRPC6.[35] Triggers of cation channel activity include oxidative stress.[44] As shown previously,[39,45,46] increased cytosolic Ca^{2+} activity stimulates cell membrane scrambling leading to phosphatidylserine exposure at the erythrocyte surface. In the nominal absence of extracellular Ca^{2+}, the scrambling effect of apigenin is significantly blunted, an observation highlighting the role of Ca^{2+} entry. However, even in the nominal absence of extracellular Ca^{2+}, apigenin still significantly increased cell membrane scrambling. Thus, additional mechanisms must be operative during apigenin induced eryptosis.

Cell membrane scrambling is stimulated by ceramide,[40,47] which is similarly known to trigger apoptosis of nucleated cells.[48] As a matter of fact, apigenin significantly increased ceramide abundance. Thus, ceramide formation presumably contributes to the stimulation of cell membrane scrambling.

Eryptosis is further triggered by energy depletion.[41] Apigenin indeed decreased cytosolic ATP concentration, an effect again contributing to the stimulation of eryptosis. The effect of apigenin on cytosolic ATP concentration could result from enhanced ATP utilization or impaired formation of ATP by interference with glycolytic flux. In theory, ATP depletion could result from Ca^{2+} entry with energy consuming export of Ca^{2+}. However, removal of extracellular Ca^{2+} did not blunt the decline of cytosolic Ca^{2+} following apigenin treatment.

Apigenin led, in addition to its effect on cell membrane scrambling, to cell shrinkage, an effect most likely resulting from activation of Ca^{2+} sensitive K^+ channels by increase of cytosolic Ca^{2+} activity.[37,49] The opening of those channels leads to K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with osmotically obliged water.[38] In theory, apigenin could, in addition, activate Cl^- channels. The present observations do, however, not allow any safe conclusions on Cl^- channel activity.

Apigenin shares its proeryptotic activity with a wide variety of xenobiotics similarly triggering eryptosis.[50−57] Morover, eryptosis is enhanced in diverse clinical disorders,[34] including diabetes,[58−60] renal insufficiency,[61] hemolytic uremic syndrome,[62] sepsis,[63] sickle cell disease.[64] malaria,[65−69] Wilson's disease,[69] iron deficiency,[70] phosphate depletion[71] and presumably metabolic syndrome.[72]

As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, excessive eryptosis may lead to anemia.[34] Moreover, phosphatidylserine-exposing erythrocytes may impair microcirculation by adherence to the vascular wall[73−77] and by stimulating blood clotting.[73,78,79]

In conclusion, apigenin triggers Ca^{2+} entry, ceramide formation and ATP depletion, effects eventually leading to eryptosis, characterized by cell shrinkage and cell membrane scrambling of erythrocytes.

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