JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7591–7595 7591
DOI:10.1021/jf900773w

Inhibition of Suicidal Erythrocyte Death by Xanthohumol

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Xanthohumol is a proapoptotic hop-derived beer component with anticancer and antimicrobial activities. Similar to nucleated cells, erythrocytes may undergo suicidal cell death or eryptosis, which is triggered by oxidative stress (*tert*-butylhydroperoxide, TBOOH) or energy depletion (removal of glucose). The triggers increase cytosolic Ca^{2+} concentration, leading to activation of Ca^{2+}-sensitive K^{+} channels with subsequent cell shrinkage and to cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocyte surface. Eryptotic cells are cleared from the circulating blood, leading to anemia, and may adhere to the vascular wall, thus impeding microcirculation. The present experiments explored whether xanthohumol influences eryptosis using flow cytometry. Exposure of human erythrocytes to 0.3 mM TBOOH or incubation in glucose-free solution significantly increased Fluo3 fluorescence (Ca^{2+} concentration) as well as annexin V-binding (cell membrane scrambling) and decreased forward scatter (cell volume), effects significantly blunted by xanthohumol. In conclusion, xanthohumol is a potent inhibitor of suicidal erythrocyte death in vitro.

KEYWORDS: Cell volume; eryptosis; phosphatidylserine; calcium; cell death

INTRODUCTION

Xanthohumol, the major prenylated flavonoid in beer (*1*−*3*), has previously been shown to exert anti-inflammatory, antiangiogenic, anticancer, antibacterial, antifungal, antimalarial, and antiviral activities (*4*). Beyond that, it has been proposed for the management of sleep disorders and menopausal symptoms in women (*5*). The anticancer efficacy has been attributed to inhibition of cell proliferation and stimulation of apoptosis (*6*). The antiproliferative and proapoptotic effects are at least partially due to increase in p21 and p53 expression and decreased expression of survivin. Xanthohumol may increase the cellular content of reactive oxidant species but at the same time may exert antioxidant activity (*7*). Moreover, xanthohumol has been shown to up-regulate the detoxification enzyme NADPH-quinone oxidoreductase (*8*).

Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal cell death, or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling leading to phosphatidylserine exposure at the cell surface (*9*). Cell membrane scrambling is triggered by an increase in cytosolic Ca^{2+} activity, which may result from activation of Ca^{2+}-permeable cation channels (*10*, *11*). The Ca^{2+} sensitivity of phospholipid scrambling is enhanced by ceramide. Increased cytosolic Ca^{2+} activity is further followed by the activation of Ca^{2+}-sensitive K^{+} channels with subsequent exit of KCl and osmotically obliged water, leading to cell shrinkage. Stimulators of Ca^{2+} entry and eryptosis include energy depletion (*12*) and oxidative stress (*9*).

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The present study explored the influence of xanthohumol on eryptosis. We demonstrate that xanthohumol blunts the increase in cytosolic Ca^{2+}, cell membrane scrambling, and cell shrinkage following energy depletion as well as the increase in cytosolic Ca^{2+} and cell membrane scrambling following oxidative stress.

MATERIALS AND METHODS

Erythrocytes, Solutions, and Chemicals. Experiments were performed at 37 °C with banked erythrocytes provided by the blood bank of the University of Tübingen. The study was approved by the ethics committee of the University of Tübingen (184/2003 V).

Erythrocytes were incubated at a hematocrit of 0.4% in 200 μL of Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 mM glucose, and 1 mM $CaCl_2$, pH 7.4, at 37 °C for the indicated time periods. When stated, glucose was deleted from the medium or *tert*-butylhydroperoxide (TBOOH; 0.3 mM, Sigma, Schnelldorf, Germany) added. Xanthohumol (Sigma) was used at concentrations between 0.25 and 1 μM, caffeine (Sigma) at 500 μM, and *N*-acetylcysteine (Sigma) at 500 μM.

FACS Analysis of Annexin V Binding and Forward Scatter. Fluorescence-activated-cell-sorter (FACS) analysis was performed as described previously (*13*). After incubation under the respective experimental condition, 50 μL of the cell suspension was used for staining. The cells were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-Fluos (Roche, Mannheim, Germany) in this solution for 20 min under protection from light. The forward scatter 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). A total of 15000−25000 erythrocytes were counted per experiment.

Measurement of Intracellular Ca^{2+}. After incubation under the respective experimental condition, 50 μL of the cell suspension was used

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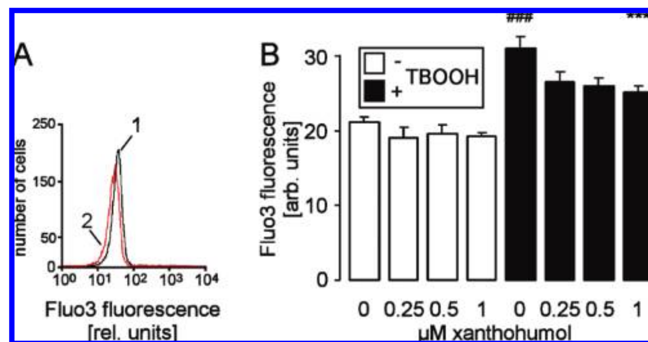

Figure 1. Effect of oxidative stress on cytosolic Ca^{2+} activity in the presence and absence of xanthohumol: (**A**) histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 30 min with 0.3 mM *tert*-butylhydroperoxide (TBOOH) in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means ± SEM (n = 8−20) of Fluo3 fluorescence in erythrocytes following incubation for 30 min in the absence (white bars) or presence (black bars) of TBOOH (0.3 mM) in the absence or presence of xanthohumol [### ($p < 0.001$) indicates significant difference from the absence of TBOOH; *** ($p < 0.001$) indicates significant difference from the absence of xanthohumol (ANOVA)].

for staining. The erythrocytes were washed in Ringer solution and then loaded with Fluo3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. The cells were incubated at 37 °C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo3/AM-loaded erythrocytes were resuspended in 200 μL of Ringer solution. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis (exciation wavelength, 488 nm; emission wavelength, 530 nm).

Statistics. Data are expressed as arithmetic means ± SEM, and statistical analysis was made using ANOVA with Tukey's test as post test. n denotes the number of different erythrocyte specimens tested.

RESULTS

In a first series of experiments, the effect of oxidative stress on erythrocytes in the absence and presence of xanthohumol was elucidated. Fluo3 fluorescence was utilized to determine cytosolic Ca^{2+} activity. As illustrated in **Figure 1**, a 30 min exposure of human erythrocytes to TBOOH (0.3 mM) was followed by a marked, statistically significant increase in Fluo3 fluorescence. The increase in Fluo3 fluorescence in the presence of 0.3 mM TBOOH was blunted in the presence of 1 μM xanthohumol. In another series of experiments, the potency of xanthohumol to inhibit an increase in cytosolic Ca^{2+} activity upon oxidative stress was compared to that of *N*-acetylcysteine, a known scavenger for oxidative stress. As a result, exposure of erythrocytes to 0.3 mM TBOOH resulted in a Fluo3 fluorescence of (all arbitrary units) 30.52 ± 1.25 (n = 4). The presence of 0.5 μM xanthohumol decreased Fluo3 fluorescence to 25.76 ± 2.13 (n = 4), whereas the presence of 0.5 mM *N*-acetylcysteine decreased Fluo3 fluorescence to 27.17 ± 1.21 (n = 4), effects, however, not reaching statistical significance in this series.

Annexin V binding was employed to determine the percentage of phosphatidylserine-exposing erythrocytes. As shown in **Figure 2**, 0.3 mM TBOOH markedly increased annexin V binding. The effect of TBOOH on the percentage of annexin V binding cells was blunted in the presence of 0.5 μM xanthohumol. Accordingly, xanthohumol significantly blunted the stimulation of cell membrane scrambling by 0.3 mM TBOOH. Another series of experiments aimed to compare the potency of xanthohumol to that of *N*-acetylcysteine. As a result, exposure of erythrocytes to 0.3 mM TBOOH resulted in 46.9 ± 3.4% (n = 4) PS-exposing erythrocytes. The presence of 0.5 μM xanthohumol significantly

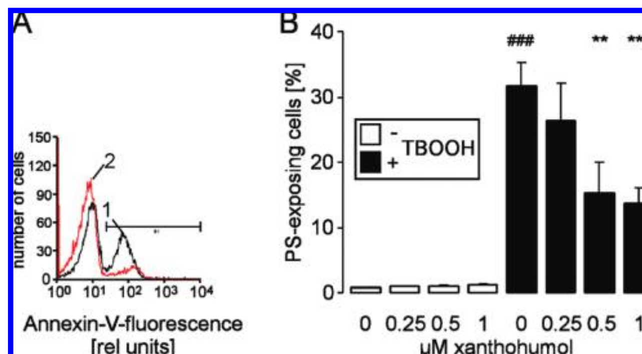

Figure 2. Effect of oxidative stress on phosphatidylserine exposure in the presence and absence of xanthohumol: (**A**) histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 30 min with 0.3 mM *tert*-butylhydroperoxide (TBOOH) in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means ± SEM (n = 8−20) of the percentage of annexin V binding erythrocytes following incubation for 30 min in the absence (white bars) or presence (black bars) of TBOOH (0.3 mM) in the absence or presence of xanthohumol [### ($p < 0.001$) indicates significant difference from the absence of TBOOH; ** and *** ($p < 0.01$ and $p < 0.001$) indicate significant difference from the absence of xanthohumol (ANOVA)].

($p < 0.05$) decreased the percentage of PS-exposing erythrocytes to 25.4 ± 5.9% (n = 4), whereas the presence of 0.5 mM *N*-acetylcysteine significantly ($p < 0.05$) decreased the percentage of PS-exposing erythrocytes to 33.8 ± 2.0% (n = 4).

To determine the effect of oxidative stress on cell volume in the presence and absence of xanthohumol, the forward scatter was determined in FACS analysis. As illustrated in **Figure 3A**,**B**, TBOOH (0.3 mM) significantly decreased the erythrocyte forward scatter. The effect of TBOOH on forward scatter was not appreciably modified by xanthohumol (**Figure 3**). Cell shrinkage was further analyzed by defining a population of shrunken erythrocytes in FACS analysis (**Figure 3A**). This analysis confirmed the results retrieved from **Figure 3B** (see **Figure 3C**). Another series of experiments aimed to compare the potency of xanthohumol to *N*-acetylcysteine. As a result, exposure of erythrocytes to 0.3 mM TBOOH resulted in a forward scatter of (all arbitrary units) 328.9 ± 19.2 (n = 4). The presence of 0.5 μM xanthohumol increased the forward scatter to 366.6 ± 20.3 (n = 4), whereas the presence of 0.5 mM *N*-acetylcysteine increased the forward scatter to 355.4 ± 9.5 (n = 4), effects, however, not reaching statistical significance in this series.

In a further series of experiments, the effect of energy depletion was determined in the absence and presence of xanthohumol. As shown in **Figure 4**, a 48 h incubation of erythrocytes in the absence of glucose significantly increased the Fluo3 fluorescence. The addition of xanthohumol blunted the increase in Fluo3 fluorescence following glucose depletion, an effect reaching statistical significance at 0.5 μM xanthohumol concentration. Thus, glucose depletion increased the cytosolic Ca^{2+} activity, an effect blunted by xanthohumol (≥0.5 μM). In another series of experiments, the potency of xanthohumol to inhibit an increase in the cytosolic Ca^{2+} activity upon energy depletion was compared to that of caffeine, a known inhibitor of eryptosis during energy depletion. As a result, exposure of erythrocytes to glucose-free Ringer solution resulted in a Fluo3 fluorescence of (all arbitrary units) 38.72 ± 1.78 (n = 8). The presence of 0.5 μM xanthohumol significantly ($p < 0.001$) decreased Fluo3 fluorescence to 27.71 ± 2.68 (n = 8), whereas the presence of 500 μM caffeine significantly ($p < 0.001$) decreased Fluo3 fluorescence to 29.49 ± 1.60 (n = 8).

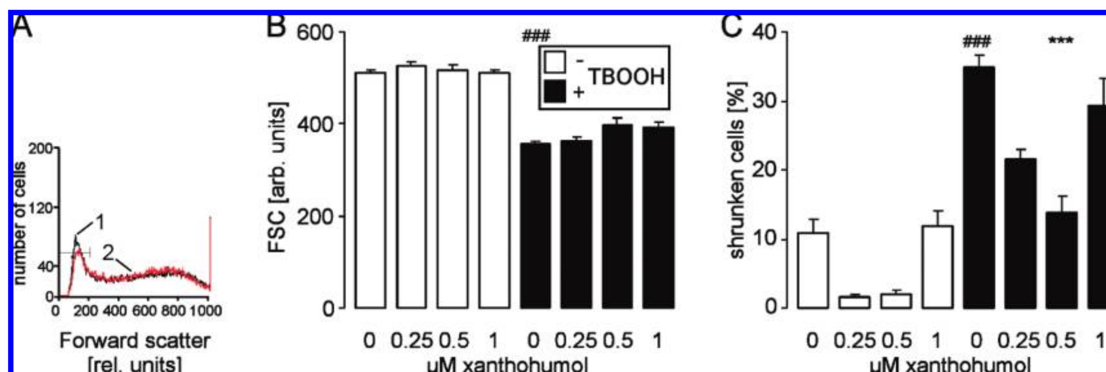

Figure 3. Effect of oxidative stress on erythrocyte forward scatter in the presence and absence of xanthohumol: (**A**) histogram of erythrocyte forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 30 min with 0.3 mM *tert*-butylhydroperoxide (TBOOH) in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means ± SEM ($n = 8-20$) of the erythrocyte forward scatter following incubation for 30 min in the absence (white bars) or presence (black bars) of TBOOH (0.3 mM) in the absence or presence of xanthohumol [### ($p < 0.001$) indicates significant difference from absence of TBOOH (ANOVA)]; (**C**) arithmetic means ± SEM ($n = 8-20$) of the percentage of shrunken erythrocytes following incubation for 30 min in the absence (white bars) or presence (black bars) of TBOOH (0.3 mM) in the absence or presence of xanthohumol [### ($p < 0.001$) indicates significant difference from the presence of glucose; *** ($p < 0.001$) indicates significant difference from the absence of xanthohumol (ANOVA)]. The percentage of shrunken erythrocytes was determined by means of the marker shown in **A**.

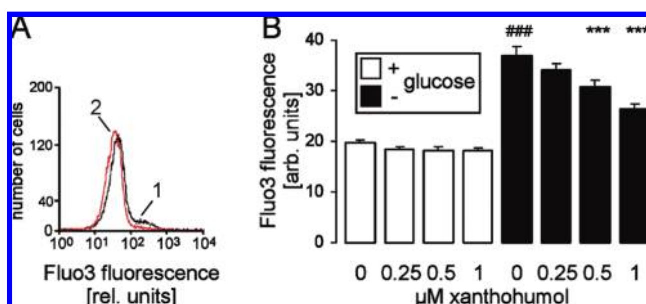

Figure 4. Effect of glucose depletion on cytosolic Ca^{2+} activity in the presence and absence of xanthohumol: (**A**) histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48 h without glucose in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means ± SEM ($n = 10$) of Fluo3 fluorescence in erythrocytes following incubation for 48 h in the presence (white bars) or absence (black bars) of glucose in the absence (0) or presence of 0.25−1 μM xanthohumol [### ($p < 0.001$) indicates significant difference from the presence of glucose; *** ($p < 0.001$) indicates significant difference from the absence of xanthohumol (paired ANOVA)].

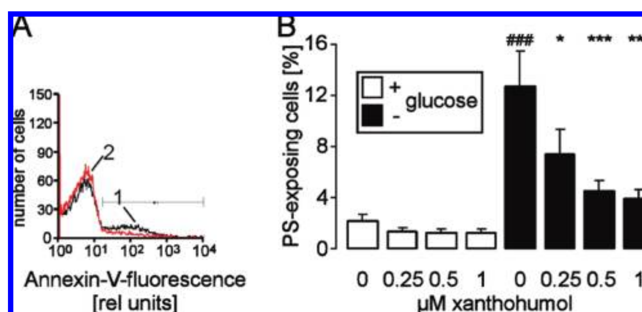

Figure 5. Stimulation of phosphatidylserine exposure by glucose depletion in the presence and absence of xanthohumol: (**A**) histogram of erythrocyte annexin V binding in a representative experiment of erythrocytes from healthy volunteers incubated for 48 h without glucose in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means ± SEM ($n = 10$) of the percentage of phosphatidylserine-exposing erythrocytes following incubation for 48 h in the presence (white bars) or absence (black bars) of glucose in the absence (0) or presence of 0.25−1 μM xanthohumol [### ($p < 0.001$) indicates significant difference from the presence of glucose; * and *** ($p < 0.05$ and $p < 0.001$) indicate significant difference from the absence of xanthohumol (paired ANOVA)].

Glucose depletion further triggered cell membrane scrambling as evident from phosphatidylserine exposure. As illustrated in **Figure 5**, glucose depletion significantly increased annexin V binding. Xanthohumol decreased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 0.25 μM xanthohumol (**Figure 5**). Thus, energy depletion stimulated cell membrane scrambling, an effect significantly blunted by xanthohumol (≥0.25 μM). Another series of experiments aimed to compare the potency of xanthohumol to caffeine. As a result, a 48 h exposure of erythrocytes to glucose-free Ringer solution resulted in 18.6 ± 1.3% ($n = 8$) PS-exposing erythrocytes. The presence of 0.5 μM xanthohumol significantly ($p < 0.001$) decreased the percentage of PS-exposing erythrocytes to 4.0 ± 0.4% ($n = 8$), whereas the presence of 500 μM caffeine significantly ($p < 0.001$) decreased the percentage of PS-exposing erythrocytes to 8.8 ± 1.5% ($n = 8$).

Glucose depletion was further followed by a decrease of erythrocyte forward scatter in FACS analysis, reflecting cell shrinkage (**Figure 6A**,**B**). The effect was blunted in the presence of xanthohumol. For a statistically significant effect, xanthohumol concentrations of ≥0.5 μM were required. Cell shrinkage was further analyzed by defining a population of shrunken erythrocytes in FACS analysis (**Figure 6A**). This analysis confirmed the results retrieved from forward scatter (**Figure 6C**). Thus, xanthohumol blunted the cell shrinkage following energy depletion. Another series of experiments aimed to compare the potency of xanthohumol to caffeine. As a result, a 48 h exposure of erythrocytes to glucose-free Ringer solution resulted in a forward scatter (all arbitrary units) of 158.8 ± 10.5 ($n = 8$). The presence of 0.5 μM xanthohumol significantly ($p < 0.001$) increased the forward scatter to 364.3 ± 40.0 ($n = 8$), whereas the presence of 500 μM caffeine significantly ($p < 0.01$) increased the forward scatter to 314.6 ± 26.0 ($n = 8$).

DISCUSSION

As reported earlier (*9*), oxidative stress and glucose depletion increase erythrocyte Ca^{2+} activity with subsequent stimulation of

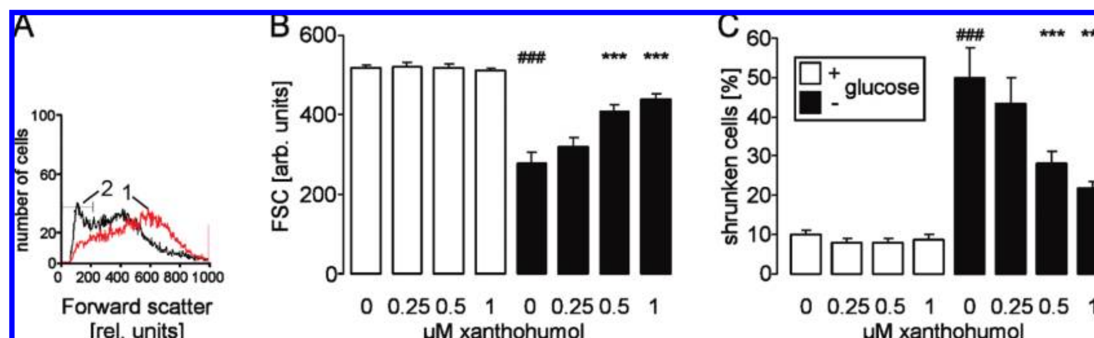

Figure 6. Forward scatter prior to and following glucose depletion in the presence and absence of xanthohumol: (**A**) histogram of erythrocyte forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 48 h without glucose in the absence (1, black line) and presence (2, red line) of 1 μM xanthohumol; (**B**) arithmetic means $\pm$ SEM ($n = 12$) of the forward scatter of erythrocytes following incubation for 48 h in the presence (white bars) or absence (solid bars) of glucose in the absence (0) or presence of 0.25−1 μM xanthohumol [### ($p < 0.001$) indicates significant difference from the presence of glucose; $***$ ($p < 0.001$) indicates significant difference from the absence of xanthohumol (paired ANOVA)]; (**C**) arithmetic means $\pm$ SEM ($n = 10$) of the percentage of shrunken erythrocytes following incubation for 48 h in the presence (white bars) or absence (solid bars) of glucose in the absence (0) or presence of 0.25−1 μM xanthohumol [### ($p < 0.001$) indicates significant difference from the presence of glucose; $***$ ($p < 0.001$) indicates significant difference from the absence of xanthohumol (paired ANOVA)]. The percentage of shrunken erythrocytes was determined by means of the marker shown in **A**.

cell membrane scrambling and erythrocyte cell shrinkage. Accordingly, both oxidative stress and energy depletion trigger eryptosis, the suicidal death of erythrocytes. The present observations disclose the powerful protective effect of xanthohumol against eryptosis following oxidative stress and energy depletion. The xanthohumol content of beer may approach up to 0.2 mg/L, corresponding to 0.6 μM (*14*). The ingestion and equal distribution of 1 L of beer in extracellular space would thus yield some 30 nM. Moreover, xanthohumol may result from conversion of precursors by intestinal bacteria (*15*). However, the bioavailability of xanthohumol is limited, and only a small fraction of the substance may enter the blood and interact with erythrocytes (*16*−*19*).

The effects of xanthohumol on Fluo3 and forward scatter were significantly different between energy depletion and oxidative stress. The differences may have been due to xanthohumol-insensitive mechanisms triggered by oxidative stress, due to partial inactivation of xanthohumol by oxidation or due to the more pronounced eryptosis following oxidative stress, which may become more difficult to reverse. In any case, xanthohumol decreased eryptosis following either oxidative stress or energy depletion, pointing to involvement of xanthohumol-sensitive mechanisms in both types of suicidal erypthrocyte death.

The increase in cytosolic Ca^{2+} activity following oxidative stress is due to stimulation of Ca^{2+}-permeable cation channels, the cell shrinkage due to subsequent activation of Ca^{2+}-sensitive K^+ channels with exit of KCl and osmotically obliged water. Ca^{2+} further stimulates cell membrane scrambling.

The effect of xanthohumol may be exploited for the treatment of anemia due to enhanced eryptosis. Phosphatidylserine-exposing cells bind to macrophages and are subsequently engulfed and degraded (*20*). Accordingly, phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood . Eryptosis contributes to several clinical conditions associated with anemia (*9*), such as iron deficiency, phosphate depletion, hemolytic uremic syndrome, sepsis, malaria (*21*−*23*), and Wilson's disease. Moreover, eryptosis is stimulated by a wide variety of endogeneous substances and xenobiotics (*9*) including cordycepin (*24*), amyloid peptides (*25*), lipopetides (*26*), retinoic acid (*27*), amantadine, ciglitazone (*28*), cyclosporine, curcumin (*29*), valinomycin (*30*), listeriolysin, copper, cadmium (*31*), and selenium (*32*). At least in theory, xanthohumol may counteract the anemia in those clinical conditions and following exposure to the respective xenobiotics.

Beyond its role in erythrocyte turnover, eryptosis may affect microcirculation, as eryptotic erythrocytes adhere to the vascular wall (*33*). Moreover, phosphatidylserine exposure stimulates blood clotting (*34*, *35*). Eryptosis has thus been suggested to participate in the vascular injury of metabolic syndrome (*36*).

Besides its effect on erythrocytes, xanthohumol may affect the survival and function of nucleated cells (*4*,*6*). It has been shown to influence the proliferation and migration of tumor cells, an effect remarkably enhanced by hypoxia, that is, by energy depletion (*37*) At concentrations of 2−10 μM xanthohumol may induce quinone reductase in mouse hepatoma cells and has thus potential activity in the prevention of cancer (*38*). The antieryptotic effect of xanthohumol requires apparently much lower concentrations than the cytotoxic effects on tumor cells. It may be interesting to explore whether, at concentrations of xanthohumol similarly low as in the present study, xanthohumol rather protects against suicidal death of nucleated cells.

In conclusion, xanthohumol inhibits suicidal erythrocyte death following oxidative stress and energy depletion in vitro. The present study thus reveals a novel effect of this nutrient, which may well be employed in the treatment or prophylaxis of anemia and disorders of microcirculation and coagulation.

ABBREVIATIONS USED

ANOVA, analysis of variance; FACS, fluorescence-activated-cell-sorter; FL, fluorescence channel; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; TBOOH, *tert*-butylhydroperoxide.

SAFETY

There are no special safety issues for this paper.

ACKNOWLEDGMENT

We acknowledge the meticulous preparation of the manuscript by Tanja Loch.

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Received March 6, 2009. Revised manuscript received May 12, 2009. Accepted July 15, 2009. This study was supported by the Carl-Zeiss-Stiftung and the Deutsche Forschungs-gemeinschaft, Nr. La 315/13-3.