Original Paper

Inhibition of Ca²⁺ Entry and Suicidal Erythrocyte Death by Naringin

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Key Words

Phosphatidylserine • Naringin • Calcium • Cell Volume • Eryptosis

Abstract

Naringin is a dietary flavonoid from citrus fruits with antioxidant and antiapoptotic activity. Similar to apoptosis of nucleated cells, suicidal death of erythrocytes or eryptosis is paralleled by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Eryptosis is triggered by increased cytosolic Ca²⁺ activity, e.g. following energy depletion or oxidative stress. The present study thus explored whether naringin interferes with eryptosis. To this end, the cytosolic Ca²⁺ concentration was estimated from Fluo3 fluorescence, phosphatidylserine exposure from annexin-V-binding and cell volume from forward scatter in FACS analysis. As a result, energy depletion (48 h glucose removal) and oxidative stress (30 min exposure to 0.3 mM tert-butylhydroperoxide) increased Fluo-3 fluorescence, decreased the erythrocyte forward scatter and enhanced the percentage of annexin-V-binding erythrocytes. Naringin (up to 40 µM) did not significantly modify Fluo-3 fluorescence, erythrocyte forward scatter or annexin-V-binding in the presence of glucose and absence of oxidative stress. Naringin, however, significantly blunted the effect of glucose depletion and oxidative stress on Fluo-3 fluorescence, erythrocyte forward scatter or annexin-V-binding. In conclusion, naringin blunts the increase of cytosolic Ca²⁺ concentration, the shrinkage, the cell membrane scrambling and thus the suicidal death of erythrocytes following energy depletion or oxidative stress.

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Introduction

Naringin is a flavonoid from citrus fruits contributing to the bitter taste of grape fruits [1-3]. Effects of naringin include neuroprotection [4], inhibition of inflammation [3], decrease of blood lipids [5], and inhibition of bone resorption [6]. Naringin is partially effective by counteracting apoptosis [4, 7-13]. Naringin particularly protects against oxidation [14].

Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage, hallmarks of apoptotic cell death [15]. Eryptosis may be triggered by Ca^{2+} entry through Ca^{2+} -permeable cation channels [16, 17], which is followed by increase of intraerythrocytic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), activation of Ca^{2+} -sensitive K^+ channels [18], K^+ exit, hyperpolarization, Cl^- exit, cellular KCl loss accompanied by osmotically obliged water and thus by cell shrinkage [19]. An increased cytosolic Ca^{2+} activity further leads to cell membrane scrambling with breakdown of phosphatidylserine asymmetry of the cell membrane and phosphatidylserine exposure at the cell surface [20]. The cell membrane scrambling machinery of erythrocytes may further be activated by ceramide formation [21, 22], energy depletion [23] and caspase activation [24-28]. Cell membrane scrambling is further regulated by the AMP activated kinase AMPK [17], cGMP-dependent protein kinase [29] and Janus-activated kinase JAK3 [30].

Phosphatidylserine exposing erythrocytes adhere to endothelial CXCL16/SR-PSO [31]. The adhesion of phosphatidylserine exposing erythrocytes to the vascular wall may compromise microcirculation [31-36]. Phosphatidylserine exposing erythrocytes could further increase the risk of thrombosis by stimulation of blood clotting [32, 37, 38]. Moreover, phosphatidylserine exposing erythrocytes are engulfed and thus cleared from circulating blood [15]. Accordingly, excessive eryptosis may lead to anemia [15].

Excessive eryptosis contributes to the pathophysiology of several clinical disorders [15], such as diabetes [28, 39, 40], renal insufficiency [41], hemolytic uremic syndrome [42], sepsis [43], sickle cell disease [44], malaria [45-48], Wilson's disease [49], iron deficiency [50], phosphate depletion [51] and presumably metabolic syndrome [52]. Eryptosis is further stimulated by a wide variety of xenobiotics [24, 30, 53-62], which may thus enhance the risk to develop thrombosis and/or anemia.

The present study explored, whether naringin at concentrations ranging from 0 to 40 μM counteracts eryptosis following erythrocyte energy depletion and/or oxidative stress.

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 (184/2003V). 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 naringin (Sigma, Germany) and/or tert-butylhydroperoxide (Enzo, Lörrach, Germany) at the indicated concentrations.

FACS analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 50 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. 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, 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 Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Fig. 1. Effect of glucose depletion on cytosolic Ca^{2+} concentration in the presence and absence of naringin. A. Histogram of Fluo-3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours with (upper panel) and without (lower panel) glucose in the absence (1, red lines) and presence (2, black lines) of 40 μM naringin. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized Fluo-3 fluorescence in erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the presence of 0 – 40 μM naringin. *** ($p < 0.001$) indicates significant difference from the presence of glucose. #, ##, ### ($p < 0.05$, $p < 0.01$, $p < 0.001$) indicate significant difference from the absence of naringin (ANOVA).

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 batches of erythrocytes may differ in their susceptibility to triggers of eryptosis, the same erythrocyte batches have been used for control and experimental conditions.

Results

In order to determine erythrocyte Ca^{2+} concentration, experiments were performed in erythrocytes loaded with the Ca^{2+} -sensitive dye Fluo-3. As illustrated in Fig. 1, cytosolic Ca^{2+} concentration was not significantly modified by exposure of erythrocytes to naringin (up to 40 μM) in energy replete erythrocytes. Energy depletion by a 48 hours exposure of the erythrocytes to glucose free extracellular fluid significantly increased cytosolic Ca^{2+} concentration (Fig. 1). In the presence of naringin, the increase in the cytosolic Ca^{2+} concentration during glucose depletion was blunted, an effect reaching statistical significance at 20 μM naringin (Fig. 1).

Increased cytosolic Ca^{2+} concentrations are expected to stimulate Ca^{2+} -sensitive K^+ channels with subsequent cell shrinkage. Thus, cell volume was estimated from forward scatter. In the presence of glucose, the addition of naringin (up to 40 μM) did not significantly modify erythrocyte forward scatter. Glucose depletion was followed by a significant decrease of erythrocyte forward scatter, an effect blunted in the presence of naringin (Fig. 2). The

Fig. 2. Effect of glucose depletion on forward scatter in the presence and absence of naringin. A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours with (upper panel) and without (lower panel) glucose in the absence (1, red lines) and presence (2, black lines) of 40 μ M naringin. B. Arithmetic means $\pm$ SEM ($n = 8$) of forward scatter in erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the presence of 0 – 40 μ M naringin. *** ($p < 0.001$) indicates significant difference from the presence of glucose. # ($p < 0.05$) indicates significant difference from the absence of naringin (ANOVA).

Fig. 3. Effect of glucose depletion on phosphatidylserine exposure in the presence and absence of naringin. A. Histogram of erythrocyte annexin-V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 48 hours with (upper panel) and without (lower panel) glucose in the absence (1, red lines) and presence (2, black lines) of 40 μ M naringin. B. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of annexin-V-binding erythrocytes following incubation for 48 hours in the presence (open bars) or absence (closed bars) of glucose in the presence of 0 – 40 μ M naringin. *** ($p < 0.001$) indicates significant difference from the presence of glucose. ##, ### ($p < 0.01$, $p < 0.001$) indicate significant difference from the absence of naringin (ANOVA).

effect of naringin on forward scatter in glucose depleted erythrocytes reached statistical significance at 40 μ M naringin concentration (Fig. 1).

Fig. 4. Effect of oxidative stress on forward scatter in the presence and absence of naringin. A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers incubated for 30 min without (upper panel) and with (lower panel) tert-butylhydroperoxide (tBOOH, 0.3 mM) in the absence (1, red lines) and presence (2, black lines) of 40 μM naringin. B. Arithmetic means $\pm$ SEM ($n = 4$) of the forward scatter in erythrocytes following incubation for 30 min in the absence (open bars) or presence (closed bars) of tert-butylhydroperoxide (0.3 mM) in the absence (0) or presence of 40 μM naringin. *** ($p < 0.001$) indicates significant difference from the absence of tert-butylhydroperoxide. # ($p < 0.05$) indicates significant difference from the absence of naringin (ANOVA).

Fig. 5. Effect of oxidative stress on phosphatidylserine exposure in the presence and absence of naringin. A. Histogram of annexin-V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 30 min without (upper panel) and with (lower panel) tert-butylhydroperoxide (0.3 mM) in the absence (1, red lines) and presence (2, black lines) of 40 μM naringin. B. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes following incubation for 30 min in the absence (open bars) or presence (closed bars) of tert-butylhydroperoxide (tBOOH, 0.3 mM) in the absence (0) or presence of 40 μM naringin. *** ($p < 0.001$) indicates significant difference from the absence of tert-butylhydroperoxide. ## ($p < 0.01$) indicates significant difference from the absence of naringin (ANOVA).

An increase in cytosolic Ca^{2+} concentration is further expected to trigger cell membrane scrambling with phosphatidylserine exposure at the cell surface. The percentage of phosphatidylserine exposing cells was estimated from annexin-V-binding. In the presence

of glucose, the percentage of annexin-V-binding erythrocytes was low and not significantly modified by naringin (up to 40 μ M). A 48 hours glucose depletion was followed by a significant increase of the percentage annexin-V-binding erythrocytes (Fig. 3). As illustrated in Fig. 3, the effect of glucose depletion on annexin-V-binding was significantly blunted in the presence of naringin (≤ 10 μ M).

In a further series of experiments, suicidal erythrocyte death was stimulated by induction of oxidative stress. To this end, the erythrocytes were exposed to 0.3 mM tert-butylhydroperoxide in Ringer solution for 30 minutes. As illustrated in Fig. 4, oxidative stress significantly decreased cell volume, an effect slightly, but significantly attenuated in the presence of naringin at 40 μ M concentration. As shown in Fig. 5, oxidative stress further significantly increased annexin-V-binding, an effect again significantly blunted in the presence of naringin (40 μ M).

Discussion

The present observations reveal a novel effect of naringin, i.e. inhibition of suicidal erythrocyte death or eryptosis. In intact erythrocytes, naringin is without appreciable effect on cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), cell volume and phosphatidylserine asymmetry of the cell membrane. As shown earlier, both, energy depletion [23] and oxidative stress [24, 28, 40, 63], enhance $[\text{Ca}^{2+}]_i$ with subsequent cell shrinkage and stimulation of cell membrane scrambling, hallmarks of both, apoptosis and eryptosis [15]. The concentration required for this effect is 10 μ M (approximately 58 mg/kg). In animals dosages ranging between 30 to 80 mg/kg have been used [64, 65].

The interference of naringin with increase of cytosolic Ca^{2+} activity may be related to an antioxidant action of naringin, as the erythrocyte cation channels are activated by oxidative stress [66]. The molecular identity of the channels has remained elusive, but the channels may involve the Ca^{2+} permeable cation channels TRPC6 [16].

Increase of cytosolic Ca^{2+} activity following energy depletion and/or oxidative stress is followed by activation of Ca^{2+} sensitive K^+ channels [18, 67], resulting in K^+ exit following its chemical gradient, cell membrane hyperpolarisation and thus potential driven Cl^- exit. The cellular KCl loss together with osmotically obliged water thus leads to cell shrinkage [19]. The inhibitory effect of naringin on the erythrocyte shrinkage following energy depletion and oxidative stress is presumably the result of its inhibitory effect on Ca^{2+} entry.

The inhibitory effect of naringin on the increase of cytosolic Ca^{2+} activity following energy depletion and/or oxidative stress further counteracts cell membrane scrambling and phosphatidylserine exposure at the erythrocyte surface, a well known feature of suicidal erythrocyte death [20, 68, 69].

In conclusion, the exposure of erythrocytes to energy depletion or oxidative stress leads to Ca^{2+} entry with subsequent triggering cell membrane scrambling and cell shrinkage, effects all blunted by naringin. Thus, at least in theory, naringin intake may favourably influence disorders associated with excessive eryptosis.

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