J Membrane Biol (2009) 230:163–171
DOI 10.1007/s00232-009-9197-x

Influences of Flavonoids on Erythrocyte Membrane and Metabolic Implication Through Anionic Exchange Modulation

Davide Barreca · Giuseppina Laganà · Ester Tellone · Silvana Ficarra · Ugo Leuzzi · Antonio Galtieri · Ersilia Bellocco

Received: 7 May 2009 / Accepted: 6 August 2009 / Published online: 1 September 2009

Abstract The antioxidative activity of some natural flavonoids was analyzed against the stable free radical 2,2-diphenyl-1-picryhydrazyl. The results indicate that the scavenging power of the tested flavonols is higher than that of the synthetic antioxidants butylated hydroxyanisole and butylated hydroxytoluene; instead, the flavanones show little activity, as indicated by efficient concentration (EC_{50}) values. Flavonoid autoxidation and interaction with Fe^{2+} and hydrogen peroxide were tested using erythrocyte membranes as a model. The results show that some compounds, like hesperetin, evidence a pro-oxidant activity higher than the ascorbic acid/iron reference system. The compounds with strong oxidative capability do not only influence cellular redox balance but also activate caspase-3, producing lactate dehydrogenase release and enhancing anionic exchange at the level of band 3 protein.

Keywords Membrane integrity · Band 3 protein · Antioxidant · Erythrocyte · Caspase-3 · Hydrogen peroxide · Metabolic modulation

Introduction

Flavonoids are polyphenolic compounds that are widespread in the human diet, being found in vegetables, fruits, herbs, tea and wine as secondary metabolites. They can be found in the free state (aglycone) or linked with sugar (glycoside). Aerobic organisms undergo oxidative modification of DNA, proteins, lipids and small molecules due to reactive oxygen species (ROS). Cells develop a variety of strategies to protect themselves against ROS, which include enzymatic and nonenzymatic scavengers. The intracellular balance between oxidants and antioxidants is very important for the homeostasis of organisms. Cardiovascular (Witztum 1993) and neurodegenerative (Frlich and Riederer 1995) disease, cancer (Borek 1991) and ischemia involve perturbation or unbalancing in this ratio. Secondary metabolites could be therapeutically utilized as antioxidants to reduce free radical–induced tissue injury. This power has encouraged investigators to search for compounds with strong antioxidant activity but low cytoxicity. Many studies have shown that diets rich in flavonoids are associated with lower risk for cardiovascular disease and anti-inflammatory and anti-allergic processes (Middleton et al. 2000; Kris-Etherton and Keen 2002). Using in vitro systems, some authors have reported that isolated flavonoids can show antioxidant and antiapoptotic activities (Rice-Evans et al. 1995; Middleton et al. 2000). Consumption of flavonoid-rich foods and beverages can be associated with increased plasma antioxidant ability, reduced platelet aggregation and improved endothelial function (Rein et al. 2000). Although these molecules have strong antioxidant activity, they can also act as pro-oxidants. This deleterious effect can be mainly due to auto- and/or enzymatic oxidation processes, through subsequent production of semiquinone and quinoidal products (Hodnick et al. 1986). Erythrocyte membrane is the widest and most used model for studying biomembrane oxidative damage (Miki et al. 1987; Mabile et al. 2001; Niki et al. 1988). In fact, it plays a fundamental role in the maintenance of intracellular microenvironment composition. Erythrocytes are vulnerable to oxidative stress due to their high content of polyunsaturated lipids, presence of oxygen molecules and transition metals (Chiu et al. 1982; Puppo and Halliwell

D. Barreca · G. Laganà · E. Tellone · S. Ficarra · U. Leuzzi · A. Galtieri (✉) · E. Bellocco
Organic and Biological Chemistry Department, University of Messina, Messina, Italy
e-mail: etellone@isengard.unime.it

1988). ROS can attack erythrocyte membrane, inducing hemolysis by oxidation of lipids and proteins (Zhu et al. 2002). The erythrocyte has several protection systems against oxidative damage (superoxide dismutase, glutathione peroxidase, catalase). The erythrocyte membrane integrity is essential for this role, so many antioxidants (such as ascorbate, β-carotene, α-tocopherol, selenium and zinc) support the enzymatic defense against oxidative damage (Roberfroid and Calderon 1995). Although the erythrocyte oxidative defense system is powerful, a remarkable oxidant insult, or certain disorders such as β-thalassemia, sickle cell anemia and glucose-6-phosphate dehydrogenase deficiency, can increase the susceptibility of the erythrocyte to peroxidation (Chiu et al. 1982). Positive or negative effects of added substances influence the functionality and integrity of erythrocytes, modulating band 3 protein activity (CD233). Band 3 (CD233) is an anion exchanger protein characterized by two structurally and functionally distinct domains: an amino-terminal 43-kDa cytoplasmic domain and a 55-kDa membrane domain. These domains are involved in the interaction with cytoskeleton proteins, hemoglobin, glycolytic enzyme, protein tyrosine kinase and the blood group antigens carbonic anhydrase II and glycophorin A. Human erythrocytes contain also inactive procaspase, which is activated by autocleavage into its active form (such as caspase-3). This activation could be the result of xenobiotic injuries but also increased levels of oxidative stress, with the resulting detachment of the N-terminal cytoplasmic domain of band 3 (cdb3) (Mandal et al. 2002, 2003; Matarrese et al. 2005; Clementi et al. 2007). The aim of this study was to analyze the behavior of these compounds, testing their abilities to scavenge 2,2-diphenyl-1-picryhydrazyl (DPPH) free radicals, to preserve erythrocyte membrane integrity, to trigger intracellular signal cascade and to influence anionic exchange at the level of band 3 protein.

Materials and Methods

DPPH Assay

The free radical–scavenging effect of compounds was assessed by the free radical method as previously reported (Blois 1958) using a stable free radical, DPPH. It forms a violet solution and reacts with antioxidants, losing color. The color loss and subsequent fall in absorbance are correlated with the antioxidant content of the sample. Tested compounds at a concentration ranging from l to 100 μM, in a final volume of 3.0 ml, were mixed with 80 μM DPPH in methanol. The changes in absorbance at 517 nm were monitored over 30 min. DPPH concentration in the cuvette was chosen to give absorbance values <1.0. The inhibition percentage of radical-scavenging activity was calculated by the following equation:

$$Inhibition(\%) = \frac{A_O - A_S}{A_O} \times 100$$

where A_O is absorbance of the control and A_S is absorbance of the sample after 30 min of incubation. From inhibition (%) was calculated the half-maximal efficient concentration (EC_{50}) by linear regression analysis. The EC_{50} value was defined as the concentration necessary to inhibit the formation of DPPH radical by 50%.

Red Blood Isolation

All donor volunteers provided written informed consent to participate in the study and were informed of all risks, discomforts and benefits involved, in accordance with the Declaration of Helsinki, for research protocols approved by the institutional review boards of the National Institutes of Health. All subjects provided medical histories, using a standardized questionnaire, and did not receive anti-inflammatory medication or nutritional supplements.

Blood was obtained by venipuncture from healthy male volunteers and collected in heparinized tubes. Erythrocytes were separated from plasma and buffy coat, washed three times with 10 volumes of 0.9% NaCl and centrifuged at 2,500 rpm for 5 min. During the last washing the packed cells were resuspended in 10 volumes of phosphate-buffered saline (PBS, pH 7.4) and utilized for the following experiments.

Assay for Erythrocyte Hemolysis

The reaction mix, in a final volume of 1.0 ml, was composed of erythrocyte (10% in PBS, pH 7.4) and 2.0 mM of different additives. It was incubated for 30 min at 37°C in a water bath and then removed, diluted with 8 volumes of PBS and centrifuged. To achieve complete hemolysis, one sample without additives was mixed with 8 volumes of distilled water and centrifuged. Another sample without additives was diluted with 8 volumes of PBS to be sure to avoid spontaneous hemolysis. After centrifugation, the absorbance of supernatant was measured at 540 nm. The results were expressed as percentage of complete hemolysis.

Interaction with H_2O_2

The interaction and oxidation process of flavonoids were evaluated also in the presence of H_2O_2. The reaction mix, in a final volume of 1.0 ml, was composed of erythrocytes (10% in PBS, pH 7.4), 100 μM of H_2O_2 and 2.0 mM of

different additives. The samples were incubated and analyzed as described in "Assay for Erythrocyte Hemolysis."

Interaction with $FeSO_4$

The reaction mix, in a final volume of 1.0 ml, was composed of erythrocytes (10% in PBS, pH 7.4), 200 μM of $FeSO_4$ and 2.0 mM of different additives. The samples were incubated and analyzed as described in "Assay for Erythrocyte Hemolysis." Ascorbic acid was used as a positive control to induce oxidative stress. The test was carried out on normal and microcythemic erythrocytes.

Kinetic Measurements of Band 3 Protein Anionic Exchange

Citrate blood samples were washed three times with an isoosmotic NaCl solution. During washing, white blood cells were discarded from the pellet. After washing, red blood cells were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM HEPES [*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid], 1.5 mM $MgCl_2$), adjusted to pH 7.4 or 7.3 and 310 ± 20 mOsmol/kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan). Cells were incubated in the above incubation buffer at 25°C in the absence or presence of 50, 100 and 200 μM of tested flavonoids or *tert*-butylhydroperoxide. At several time intervals, 10 μmol of the stopping medium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) were added to each test tube containing red blood cell suspension. Cells were then separated from the incubation medium by centrifugation (J2-HS Centrifuge; Beckman, Palo Alto, CA) and washed three times at 4°C with a sulfate-free medium to remove the sulfate trapped outside. After the last washing, the packed cells were lysed with perchloric acid (4%) and distilled water. Lysates were centrifuged for 10 min at 4,000x*g* (4°C), and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding a glycerol/distilled water mixture (1:1, v/v), 4 m NaCl, 1 m HCl and 1.23 m $BaCl_2 \cdot 2H_2O$ in order to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

The sulfate concentration was determined using a calibrated standard curve, obtained by measuring the absorbance of suspensions with known sulfate amounts (Romano et al. 1998). Experimental data of sulfate concentration as a function of the incubation time were analyzed by best-fitting procedures according to the following equation: $c(t) = c_\infty (1 - e^{-kt})$, where $c(t)$ represents sulfate concentration at time t, c_∞ the intracellular sulfate concentration at equilibrium and k the rate constant of sulfate influx.

Lactate Dehydrogenase Assay

Enzymatic activity of lactate dehydrogenase (LDH) was monitored with a UV visible spectrophotometer, following oxidation of NADH at λ_{max} of 340 nm (Murphy and Baraban 1990), during the production of lactate from pyruvate. The release of LDH was expressed in arbitrary units as a function of the control.

Caspase-3 Assay

After treatments, erythrocytes were collected, washed three times with PBS and resuspended in HEPES buffer (100 mM HEPES [pH 7.5], 20% glycerol, 5 mM DTT and 0.5 mM EDTA). Cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. The surnatant and clear lysates were passed through a Microcon YM 50 (Millipore, Bedford, MA; nominal molecular weight limit 50,000) to obtain a partial purification of caspase. The enzymatic solution was incubated with enzyme-specific colorimetric substrates (Ac-DEVD-pNA) at 37°C for 1 h. The activity of caspase-3 was analyzed with a spectrophotometer following the release of pNA at 405 nm and expressed in arbitrary units as a function of the untreated sample.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA). The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. $P < 0.05$ was regarded as indicating a significant difference.

Results

Figure 1 shows the chemical structure of the tested flavonoids. The kinetic results of the tested compounds toward DPPH radicals are shown in Fig. 2. The antioxidant activity of the analyzed flavonoids was compared to the ascorbic acid one and to the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) one. The absorbance of DPPH alone did not change over the period of the experiments. The addition of rutin, quercetin or ascorbic acid to the DPPH solution induced a rapid decrease of absorbance at 517 nm. Hesperidin's concentration, higher than that utilized in our experiments, is needed to show a considerable scavenger activity. Hesperetin reaches the highest scavenging activity at 40 μM concentration; otherwise, flavonoids like diosmin, naringenin and naringin do not show radical-scavenging activities

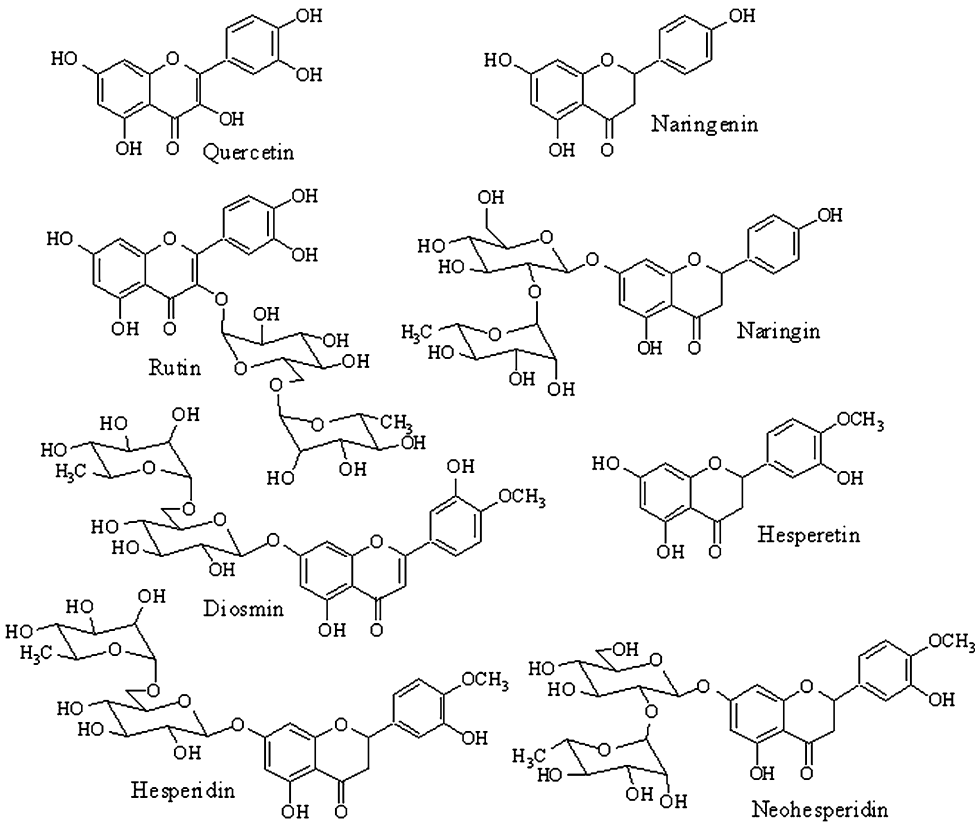

Fig. 1 Chemical structure of tested flavonoids in the free state (aglycone) or linked with sugar (glycoside)

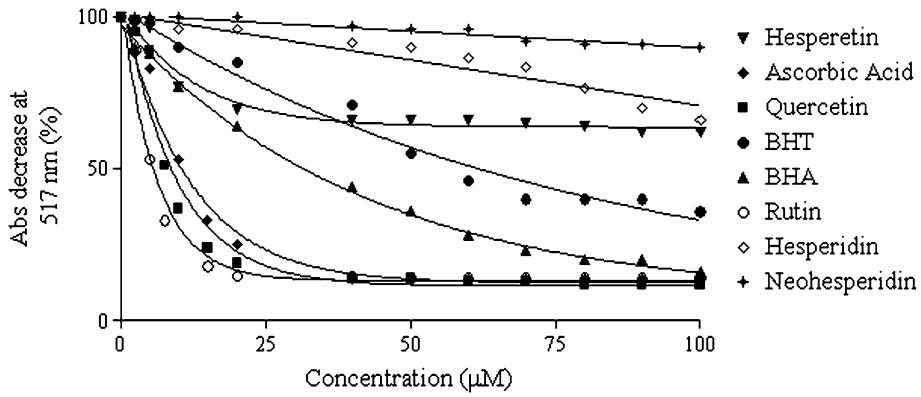

Fig. 2 Scavenger activity of tested flavonoids against DPPH radical at 517 nm, in the concentration range of 0–100 μM. Each point is the medium value, obtained with three different experiments

(data not shown). The slope of the equations is a useful parameter to define the compound's antioxidant ability. Figure 3 shows the EC_{50} values for the different additives. This graph was obtained by plotting the DPPH results as a function of the compound concentration logarithm. Flavonoids and the reference antioxidant were classified by EC_{50} values ($\leq$13, $\leq$80 and $\geq$80 μM). This assay helped us to understand if the molecules tested showed interaction with radical species but did not clarify if they acted as antioxidants or pro-oxidants. Thus, we analyzed the behavior of the compounds at the level of erythrocyte membrane, following the autoxidation process and interaction with Fe^{2+} and H_2O_2. Figure 4 shows the additives' effects on erythrocyte membrane. The results show that 2.0 mM rutin, quercetin, hesperidin, naringin or diosmin did not trigger hemolysis, unlike the same concentration of hesperetin and naringenin, which caused 30 and 26% hemolysis, respectively. Figure 5 reports the pro-oxidant activity of some compounds, analyzed as a function of their concentration (0–4 mM). The pro-oxidant activity decreased with additive concentration and caused 48 and 40% of hemolysis in the presence of 4.0 mM hesperetin and naringenin, respectively. Although erythrocyte membrane has a well-developed system to counteract H_2O_2-induced stress, its incubation in the presence of H_2O_2 (100 μM) plus 2.0 mM hesperetin and naringenin produced erythrocyte membrane damage and

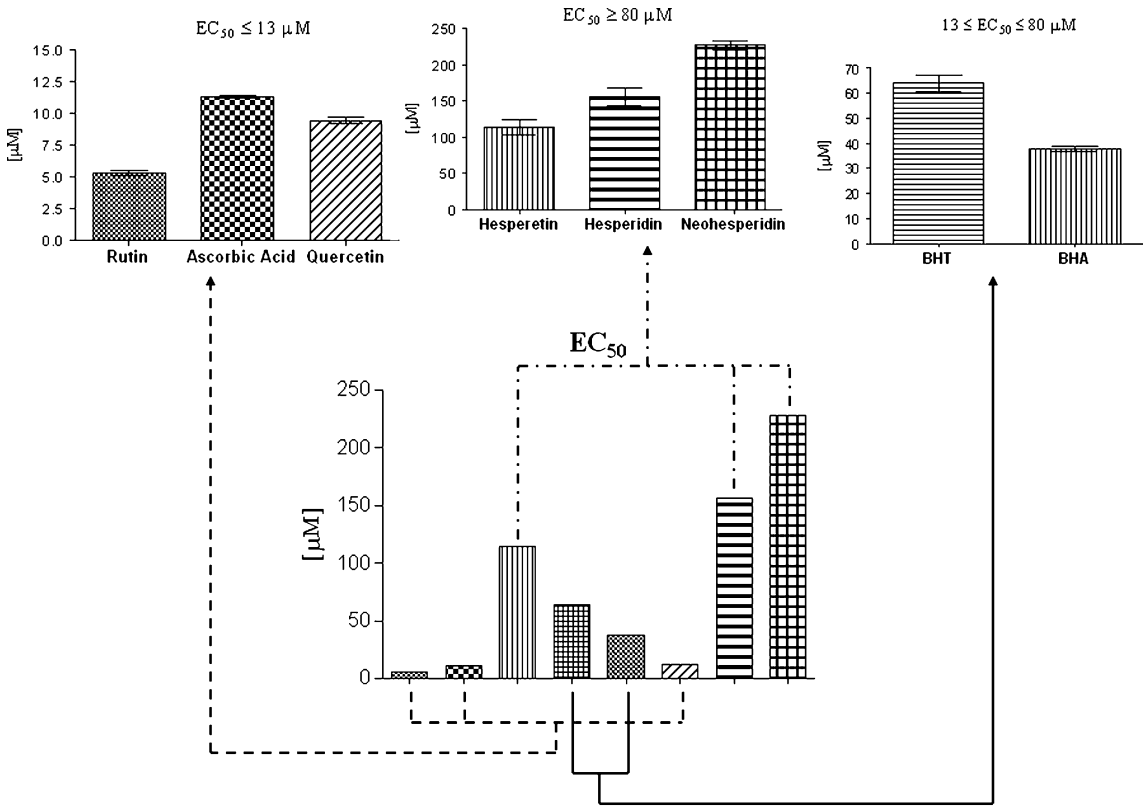

Fig. 3 Flavonoids and reference compounds efficient concentration values, obtained by plotting DPPH values as a function of the logarithm of the concentration

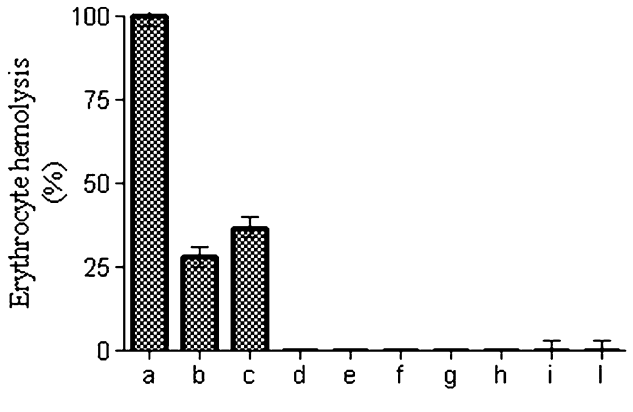

Fig. 4 Isolated erythrocytes were incubated at 37°C for 30 min in the absence or presence of 2.0 mM of the tested compounds. After incubation, samples were diluted with PBS and centrifuged. Hemolysis was analyzed as described in "Materials and Methods." *a* Complete hemolysis, *b* naringenin, *c* hesperetin, *d* ascorbic acid, *e* rutin, *f* diosmin, *g* neohesperidin, *h* quercetin, *i* hesperidin and *l* naringin. Values are means ± SD of three experiments

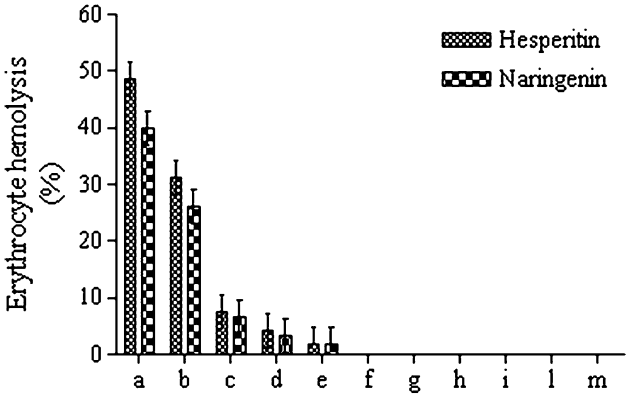

Fig. 5 Isolated erythrocytes were incubated at 37°C for 30 min in the presence of *a* 4.0, *b* 2.0, *c* 1.0, *d* 0.5, *e* 0.25, *f* 0.1, *g* 0.05, *h* 0.025, *i* 0.01, *l* 0.0025 and *m* 0.0 mM of hesperetin and naringenin. Hemolysis was analyzed as described in "Materials and Methods." Each point is the medium value, obtained with three different experiments

hemolysis up to 54 and 37%, respectively (Fig. 6). The other additives, on the contrary, did not cause any significant erythrocyte hemolysis (Fig. 6). The interaction processes of these additives with Fe^{2+} were also analyzed. The system Fe^{2+}/ascorbic acid was used as a control to evaluate oxidative stress and lipid peroxidation. The results show the same behavior for both normal and microcythemic erythrocytes, with changes that involve only the intensity of

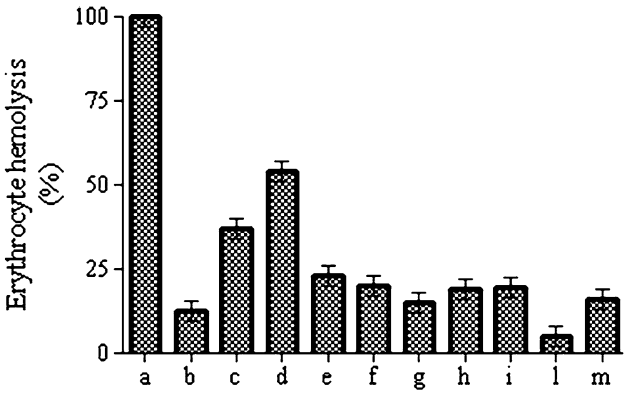

Fig. 6 Isolated erythrocytes were incubated at 37°C for 30 min in the presence of 0.1 mM of H_2O_2 with or without 2 mM of flavonoids. Hemolysis was analyzed as described in "Materials and Methods." *a* Complete hemolysis, *b* H_2O_2 + ascorbic acid, *c* H_2O_2 + naringenin, *d* H_2O_2 + hesperetin, *e* H_2O_2 + rutin, *f* H_2O_2 + diosmin, *g* H_2O_2 + hesperidin, *h* H_2O_2 + quercetin, *i* H_2O_2 + neohesperedin, *l* H_2O_2 and *m* H_2O_2 + naringin. Values are means ± SD of three experiments

action (Fig. 7). Hesperetin induced a hemolysis rate higher than Fe^{2+}/ascorbic acid system (54% in microcythemic and 39% in normal erythrocytes). Compounds like rutin, naringenin, hesperidin, diosmin and naringin with Fe^{2+} cause 5–20% of hemolysis (Fig. 7). Also, 100 μM of hesperetin and naringenin induced activation of caspase-3 (Fig. 8). On the contrary, 100 μM of the other additives did not modify caspase activity. Similar results were obtained by monitoring the release of LDH, with a statistically significant variation in the presence of hesperetin and naringenin (Fig. 8). We also analyzed the influences of the flavonoids at the level of the erythrocyte membrane in its aglycone or glycoside state. As shown in Fig. 9, the presence of *tert*-butylhydroperoxide increased the rate constant from 0.012 in the control to 0.041 and 0.169 in erythrocytes treated with 50 and 100 μM of this compound, respectively. Concentrations over 200 μM result in erythrocyte hemolysis. The same results were obtained in the presence of 50 and 100 μM of hesperetin and naringenin, with values very similar to the *tert*-butylhydroperoxide one. Erythrocyte incubation, in the presence of the other tested flavonoids, showed also an increase of the rate constant; but the variation was not so evident (Fig. 9).

Discussion

Many studies have analyzed the beneficial effects of secondary metabolites. In particular, polyphenolic compounds could show "good" or "bad" behavior, acting as antioxidants or pro-oxidants. In our experiments this double nature was analyzed using both chemical and biological assays.

Attention to antioxidants and their employment in pharmaceutical and industrial applications is growing exponentially. However, these molecules can change the intracellular balance redox and can have some negative effects for organisms. In fact, physicochemical characterization of flavonoid structures has shown that they have a redox potential between 0.23 and 0.75 *V*, suitable to reduce a large amount highly oxidized free radicals (Potapovich and Kostyuk 2003) and to interact with other important biological molecules. For example, we can analyze flavonoid redox potential as a function of ascorbate. Flavonoids containing a catechol group at the level of the B-ring and a 2,3-double bond have a higher redox potential than ascorbate and are consequently able to oxidize it to the ascorbyl radical, while flavanones are capable of reducing

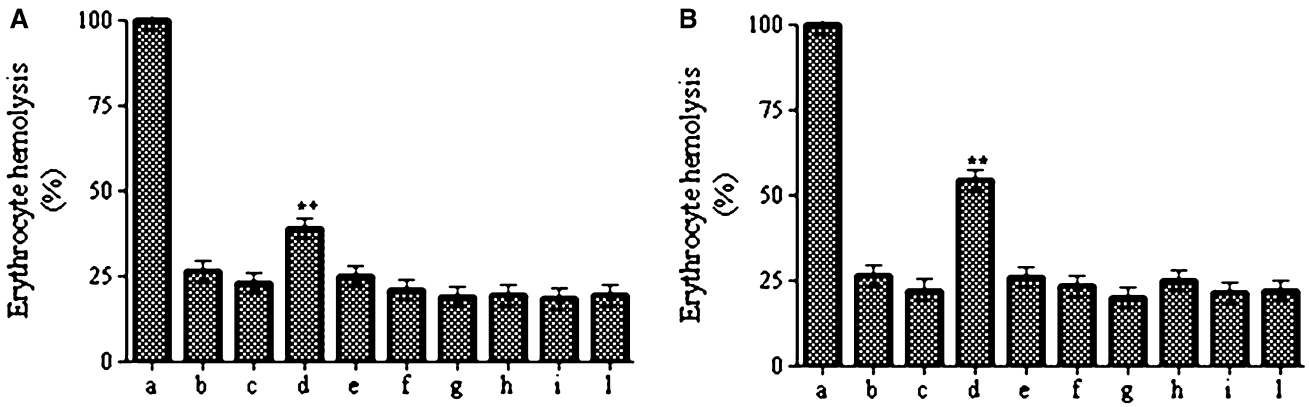

Fig. 7 Isolated erythrocytes, from normal (**a**) or microcythemic donors (**b**), were incubated at 37°C for 30 min in presence of 200 μM of $FeSO_4$ and 2.0 mM of different additives. Hemolysis was analyzed as described in "Materials and Methods." *a* Complete hemolysis, *b* $FeSO_4$ + ascorbic acid, *c* $FeSO_4$ + naringenin, *d* $FeSO_4$ + hesperetin, *e* $FeSO_4$ + rutin, *f* $FeSO_4$ + diosmin, *g* $FeSO_4$ + hesperidin, *h* $FeSO_4$ + quercetin, *i* $FeSO_4$ + neohesperedin and *l* $FeSO_4$ + naringin. Values are means ± SD of three experiments. ** Significant difference between $FeSO_4$ + ascorbic acid– and flavonoid-treated cells at $P < 0.05$

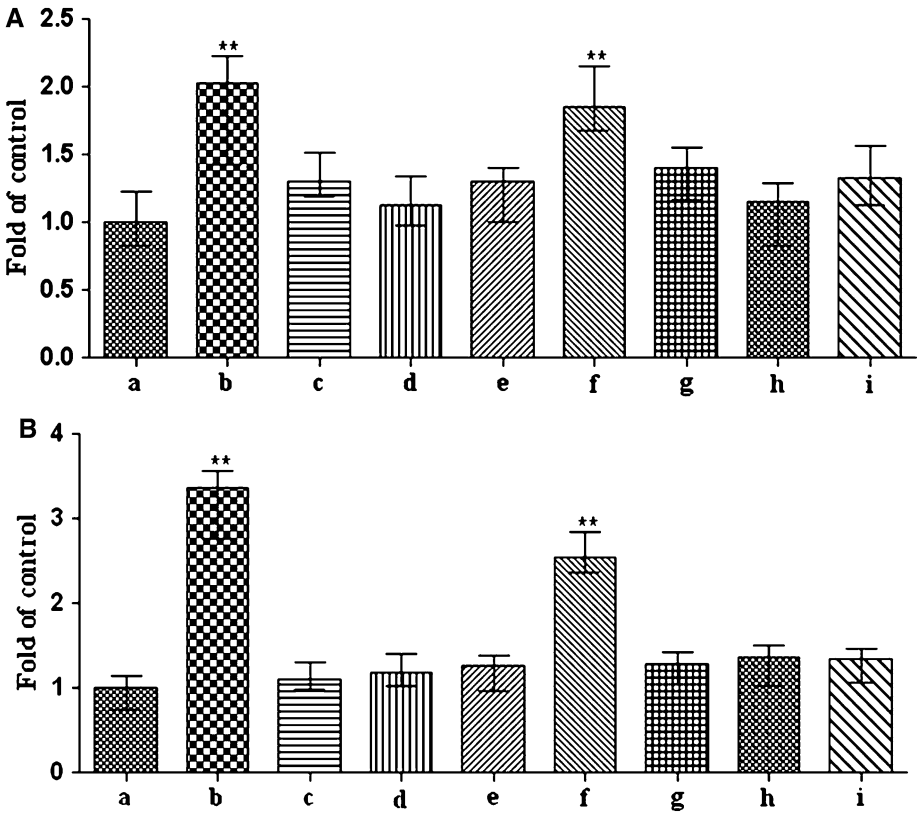

Fig. 8 Erythrocytic caspase-3 activation (**a**) and LDH release (**b**) in the presence of *a* control, *b* 100 μM of hesperetin, *c* 100 μM of hesperidin, *d* 100 μM of neohesperidin, *e* 100 μM of rutin, *f* 100 μM of naringenin, *g* 100 μM of quercetin, *h* 100 μM of diosmin and *i* 100 μM of naringin. Values are the means ± range of at least three experiments. The differences between control and flavonoid-treated erythrocytes were analyzed by one-way ANOVA, followed by Student's *t*-test. ** Significant difference between control and flavonoid-treated cells at $P < 0.05$

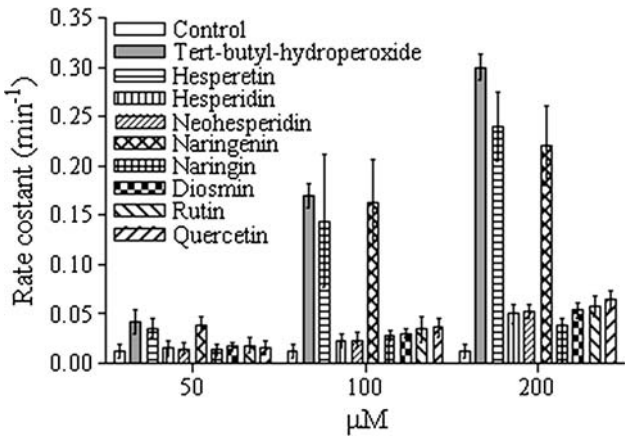

Fig. 9 Flavonoid effects on the rates of sulfate transport in normal human red blood cells. Values are the means ± SD of at least three different experiments

this molecule (Bors et al. 1995; Jovanovic et al. 1996). Taking into account all this background, we analyzed the antioxidant/pro-oxidant behavior of some flavonols and flavanones using the DPPH assay and the interaction process which lead to functional and metabolic rearrangement at the erythrocyte level. The results obtained with the DPPH assay give helpful information to understand the scavenging capacity of the compounds tested. This assay emphasizes the importance of substitutions present in the flavonol class for scavenging DPPH radicals in comparison with those in flavanones. In fact, quercetin's and rutin's radical-scavenging activity is higher than that of hesperetin, neoesperidin, hesperidin, naringin and naringenin. This assay helped us to understand if the molecules tested showed interaction with radical species but did not clarify if they act as antioxidants or pro-oxidants. Thus, we analyzed the behavior using the erythrocyte membrane as a model, following the autoxidation process and interaction with Fe^{2+} and H_2O_2. The results obtained by hemolysis assay show strong pro-oxidant action of naringenin and hesperetin, whose activity could be due to the autoxidation process. Our results show that hesperetin acts at the erythrocyte level, bringing disruption, while its glycated form (hesperidin) does not have the same effect. In accordance with other authors (Chen et al. 2003), our results confirmed the importance of A-ring C7 glycation to loss of pro-oxidant action as well as the importance of a double bond and hydroxyl group at the level of the C-ring, as in quercetin. This molecule has the same structure at the level of the A-ring, and its glycation process happened at the level of C3 (rutin). The pro-oxidant action of hesperidin in the presence of H_2O_2 could be due to the formation of hydroxyl radical. The interaction of this radical species with hesperetin makes it more reactive. The other additives help erythrocyte to protect against H_2O_2-induced stress. Also, in the presence of Fe^{2+}, hesperetin shows major pro-

oxidant activity, superior also to that of a classical model for the induction of oxidative stress (Fe^{2+}/ascorbic acid), followed by naringenin. The same results are more evident if we analyze the results obtained for microcythemic subjects. In fact, the results are the same for both donors, but in the microcythemic one they are increased in value, as expected because they are more sensible to oxidative stress. Hesperetin and naringenin induce also activation of caspase-3, the main intracellular signal for induction of the apoptotic process, and bring LDH release, showing the cytotoxic nature of the damage. Also, anionic exchanges result in strong activation by incubation in the presence of hesperetin and naringenin, and, as in the case of *t*-butyl-hydroperoxide, this could be due to an oxidative stress increase, with activation of caspase-3 at the erythrocyte level. The activation of caspase-3 causes proteolytic cleavage of cdb3 and loss of the capacity to bind glycolytic enzyme, resulting in an increase in glycolysis toward the pentose phosphate pathway, with a decrement of total erythrocytes, reducing power. In fact, the interactions at the cdb3 level are of fundamental importance because they seem to be implicated in the anion influx as in the shifting of the metabolism toward the pentose phosphate pathway or the Embden-Meyerhof-Parnas pathway (De Rosa et al. 2007; Giardina et al. 1995; Galtieri et al. 2002; Russo et al. 2008; Tellone et al. 2008).

In conclusion, our results underline the fundamental importance of substitution at the flavonoid ring level and, as in the case of hesperetin, the higher pro-oxidant nature of its flavanone skeleton, superior also to the classical model for the induction of oxidative stress (Fe^{2+}/ascorbic acid). The flavanone aglycone's remarkable activity on anion exchange proteins could be mainly due to activation of the caspase pathway, with a derangement of erythrocytic membranes (Ficarra et al. 2009) and modification of the N-terminal cytoplasmic domain of band 3. Otherwise, these molecules could interact with hemoglobin, as reported by Lu et al. (2007), modifying protein conformation and metabolic correlation with band 3 protein.

References

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181:1199–1200

Borek C (1991) Free radical processes in multistage carcinogenesis. Free Radic Res Commun 12:745–750

Bors W, Michel C, Schikora S (1995) Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: a pulse radiolysis study. Free Radic Biol Med 19:45–52

Chen Y, Shen S, Lin H (2003) Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. Biochem Pharmacol 66:1139–1150

Chiu D, Lubin B, Shohet S (1982) Peroxidative reactions in red cell biology. In: Pryor W (ed) Free radicals in biology. Academic Press, New York, pp 115–160

Clementi ME, Giardina B, Colucci D, Galtieri A, Misiti F (2007) Amyloid-beta peptide affects the oxygen dependence of erythrocyte metabolism: a role for caspase 3. Int J Biochem Cell Biol 39:727–735

De Rosa MC, Carelli Alinovi C, Galtieri A, Scatena R, Giardina B (2007) The plasma membrane of erythrocytes plays a fundamental role in the maintenance of the reduced state of the heme iron. Gene 398:162–171. doi:10.1007/s00232-008-9122-8

Ficarra S, Tellone E, Giardina B, Scatena R, Russo A, Misiti F, Clementi ME, Colucci D, Bellocco E, Laganà G, Barreca D, Galtieri A (2009) Derangment of erythrocytic AE1 in beta-thalassemia by caspase 3: phatogenic mechanisms and implication in red blood cells senescence. J Membr Biol 228:43–49. doi:10.1007/s00232-009-9157-5

Frlich I, Riederer P (1995) Free radical mechanisms in dementia of Alzheimer type and the potential for antioxidative treatment. Drug Res 45:443–449

Galtieri A, Tellone E, Romano L, Misiti F, Bellocco E, Ficarra S, Russo A, Di Rosa D, Castagnola M, Giardina B, Messana I (2002) Band 3 protein function in human erythrocytes: effect of oxygenation deoxygenation. Biochim Biophys Acta 1564:214–218

Giardina B, Messana I, Scatena R, Castagnola M (1995) The multiple functions of hemoglobin. Crit Rev Biochem Mol Biol 30:165–196

Hodnick WF, Kung FS, Roettger WJ, Bohmont CW, Pardini RS (1986) Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure–activity study. Biochem Pharmacol 35:2345–2357

Jovanovic SV, Steenken S, Hara Y, Simic MG (1996) Reduction potentials of flavonoid and model phenoxyl radicals. Which ring in flavonoids is responsible for antioxidant activity? J Chem Soc Perkin Trans 2 11:2497–2504, doi:10.1039/P29960002497

Kris-Etherton PM, Keen CL (2002) Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol 13:41–49

Lu Z, Zhang Y, Liu H, Yuan J, Zheng Z, Zou G (2007) Transport of a cancer hemopreventive polyphenol, resveratrol: interaction with serum albumin and hemoglobin. J Fluoresc 17:580–587

Mabile L, Piolot A, Boulet L, Fortin L-J, Doyle N, Rodriquez C, Davignon J, Blache D, Lussier-Cacan S (2001) Moderate intake of n-3 fatty acids is associated with stable erythrocyte resistance to oxidative stress in hypertriglyceridemic subjects. Am J Clin Nutr 74:449–456

Mandal D, Moitra PK, Saha S, Basu J (2002) Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. FEBS 513:184–188

Mandal D, Baudin-Creuza V, Bhattacharyya A, Pathak S, Delaunay J, Kundu M, Basu J (2003) Caspase 3-mediated proteolysis of the N-terminal cytoplasmic domain of the human erythroid anion exchanger 1 (band 3). J Biol Chem 278:52551–52558

Matarrese P, Straface E, Pietraforte D, Gambardella L, Vona R, Maccaglia A, Minetti M, Malorni W (2005) Peroxynitrite induces senescence and apoptosis of red blood cells through the activation of aspartyl and cysteinyl proteases. FASEB J 19:416–418

Middleton E Jr, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52: 673–751

Miki M, Tamai H, Mino M, Yamamoto Y, Niki E (1987) Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch Biochem Biophys 258: 373–380

Murphy TH, Baraban JM (1990) Glutamate toxicity in immature cortical neuron precedes development of glutamate receptor currents. Dev Brain Res 57:146–150

Niki E, Komuro E, Takahashi M, Urano S, Ito E, Terao K (1988) Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J Biol Chem 263:19809–19814

Puppo A, Halliwell B (1988) Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Biochem J 249: 185–190

Potapovich AI, Kostyuk VA (2003) Comparative study of antioxidant properties and cytoprotective activity of flavonoids. Biochemistry (Mosc) 68:514–519

Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, Keen CL (2000) Cocoa inhibits platelet activation and function. Am J Clin Nutr 72:30–35

Rice-Evans C, Nicholas J, Molwell P, Bramley P, Pridham J (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 22:375–383

Roberfroid M, Calderon PB (1995) Free radicals and oxidation phenomena in biological system. Marcel Dekker, New York

Romano L, Peritore D, Simone E, Sidoti A, Trischitta F, Romano P (1998) Chloride sulfate exchange chemically measured in human erythrocyte ghosts. Cell Mol Biol 44:351–355

Russo A, Tellone E, Ficarra S, Giardina B, Bellocco E, Laganà G, Leuzzi U, Kotyk A, Galtieri A (2008) Band 3 protein function in teleost fish erythrocytes: effect of oxygenation–deoxygenation. Physiol Res 57:49–54

Tellone E, Ficarra S, Giardina B, Scatena R, Russo A, Clementi ME, Misiti F, Bellocco E, Galtieri A (2008) Oxidative effects of gemfibrozil on anion influx and metabolism in normal and beta-thalassemic erythrocytes: physiological implications. J Membr Biol 224:1–8

Witztum JL (1993) The role of oxidized lox density lipoproteins in atherogenesis. Br Heart J 69:12–14

Zhu QY, Holt RR, Lazarus SA, Orozco TJ, Keen CL (2002) Inhibitory effects of cocoa flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis. Exp Biol Med 227:321–329