

Effect of Some Flavonic Compounds and Ascorbic Acid on Lactoferrin Stimulation of Erythrocyte Glycolysis and Na⁺/K⁺-ATPase Activity

Ana Maneva* and Borislava Taleva

Department of Biochemistry, Medical Faculty, Medical University of Sofia, Zdrave 2 Str., 1431 Sofia, Bulgaria. E-mail: a_maneva@gbg.bg

* Author for correspondence and reprint requests

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The aim of the present study was to assess if some flavonic compounds (quercetin, piceatannol and apigenin) and ascorbic acid could interfere with the Lf stimulatory effect on the erythrocyte function. Quercetin (1.5 μM) and piceatannol (30 μM) showed an additive effect on Lf stimulation of Na⁺/K⁺-ATPase when used together with Lf. The enhancement of Lf stimulation on Na⁺/K⁺-ATPase in the presence of flavonoids was probably due to their antioxidative properties and/or to their involvement in the erythrocyte signaling. None of the estimated flavonoids showed an effect on Lf stimulation of the lactate production. Quercetin itself enhanced the ATPase activity but did not affect the lactate formation. Apigenin (1.5 μM) enhanced reliably the lactate generation, but it did not exert any effect on the ATPase activity. High concentration of ascorbic acid (60 mM) did not change the Lf stimulatory effect on Na⁺/K⁺-ATPase, but decreased the Lf-specific-binding. A significantly strong inhibitory effect on the Lf-specific binding exerted the electron acceptors NAD⁺ (2 mM) and FAD (2 mM). These effects concern most likely the competition with Lf for electron(s) which is (are) provided from the erythrocyte intercellular electron transport chain(s).

Key words: Lactoferrin, Erythrocyte Glycolysis, Na⁺/K⁺-ATPase

Introduction

Lf is a metal-binding glycoprotein with antioxidative, anti-inflammatory, immunomodulatory, anticancerogenic, antibacterial, antiviral, antiatherogenic and anticoagulant properties (Levy and Viljoen, 1995). Our previous studies showed that Lf binds erythrocyte membrane receptors (Taleva *et al.*, 1999) resulting in stimulation of glycolysis, antioxidative protection (Maneva *et al.*, 2003) and activation of Na⁺/K⁺-ATPase activity (Maneva *et al.*, 2007). Lf-receptor interactions might intervene in short-term effects of regulation, involving processes of changes in association, phosphorylation and oxidation of the membrane proteins. The erythrocyte membrane contains most of the signal molecules at the beginning of the signal transduction pathway(s) (Minetti and Low, 1997), which may conduct regulatory stimuli induced by Lf.

The erythrocyte has a pool of flavonic compounds, which are considered as a buffer, maintaining the antioxidative activity of the erythrocyte (Fiorani *et al.*, 2002). Because of the application of both flavonoids and Lf for therapy and prophylaxis of a great number of diseases, it is important to study if some interactions between flavonoids and Lf exist.

Flavonoids and Lf have some common properties. They are known as scavengers of ROS (reactive oxygen species) and iron chelators (Levy and Viljoen, 1995; Middleton *et al.*, 2000). Flavonoids and Lf participate in the prevention of hemolysis (Maneva *et al.*, 2003; Dai *et al.*, 2006) and act as final acceptors of an electron transport chain, reducing the extracellular ferricyanide (Schipfer *et al.*, 1985; Maneva *et al.*, 2003) and stimulating erythrocyte glycolysis (Maneva *et al.*, 2003; Fiorani *et al.*, 2002). Both take part in the cell signal transduction as modulators of the processes of phosphorylation. Flavonoids are competitive inhibitors of many protein kinases [phosphoinositide-3-kinase (PI3K), Akt/protein kinase B (Akt/PKB), tyrosine kinase(s), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK)] (Williams *et al.*, 2004). Up to now there is no data reported whether or not Lf and flavonoids are using common signal pathways.

It has been found an electron transport chain exporting extracellular electrons generated by oxidative phosphorylation involving glyceraldehyde-3-phosphate dehydrogenase and ascorbate that activates glycolysis under physiological conditions

(Orringer and Roer, 1979). A trans-membrane ferri-cyanide oxidoreductase has been reported which is referred to the erythrocyte's ability to recover intracellular ascorbate (May and Qu, 1999). There is a theoretical possibility for the competition for electron(s) between Lf and ascorbic acid (AA).

The aim of the present study was to assess if some flavonic compounds (quercetin, piceatannol and apigenin) with broad biological activity (Middleton *et al.*, 2000) could interfere with the Lf stimulatory effect on the erythrocyte function (Maneva *et al.*, 2003, 2007). We designed our experiments in order to study whether or not the simultaneous application of both flavonoids and Lf can affect the stimulation of Lf on the glycolysis and the Na⁺/K⁺-ATPase activity. We were interested also in whether AA affects both the Lf regulation of the Na⁺/K⁺-ATPase activity and the Lf binding to the erythrocyte membrane (Taleva *et al.*, 1999). The effect of AA on the Lf binding was compared with that of the other electron acceptors, NAD⁺ and FAD.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The mentioned concentrations were chosen according the producer's prescription and the data existing about their optimal effect as follows: quercetin (1.5 μ M) is a competitive inhibitor of protein kinases and an antioxidant (Matter *et al.*, 1992); piceatannol (30 μ M) is an inhibitor of the erythrocyte signalosome (Uhie *et al.*, 2003); apigenin (1.5 μ M) is an antioxidant and inhibitor (Chaudhary and Willett, 2006).

Isolation of the erythrocytes

Heparinized fresh drawn blood from healthy donors was centrifuged at 2,000 \times g for 5 min at 4 °C, and the resulting pellet was resuspended in 4 volumes phosphate buffered saline (PBS) (0.144 g/l KH₂PO₄, 9.0 g/l NaCl, and 0.795 g/l Na₂HPO₄ · 7H₂O), pH 7.4. After three times washing at 1,800, 1,500, 1300 \times g the erythrocytes were isolated by density separation (Cohen *et al.*, 1976). The erythrocyte fraction was resuspended in PBS, pH 7.4, to obtain the same concentration as in the fresh blood. Cell concentration was counted in a Burker's camera by a Standard KF2 microscope (Carl Zeiss, Jena, Germany). The suspension did not contain other cell types.

Preparation of the erythrocyte membranes

5 ml of packed RBCs were mixed with 15 ml cold PBS, pH 7.4, and centrifuged at 5,900 \times g for 10 min at 4 °C. The supernatant was discarded, and cells were washed with 15 ml cold PBS, centrifuged as above, and resuspended in 5 ml of 5 mM Na₂HPO₄, pH 8.0, for hypotonic cell lysis. Lysed cells were centrifuged at 25,000 \times g for 15 min at 4 °C, after which the supernatant was gently aspirated and discarded. The resulting RBC membrane pellet was repeatedly washed (4–5 times) with 5 mM Na₂HPO₄ until the pellet appeared white (indicating the removal of Hb), and used for further experiments. The protein content of hemoglobin-free pellets was determined according to Bradford (1976), with human serum albumin as a calibrator. Samples were diluted to protein concentrations of 1.5 g/l.

Cell treatment

50 μ l erythrocyte suspension (2 · 10⁷ cells/ml) were incubated 30 min at 25 °C. Treatments were performed in quadruplicate. Each sample contained 50 nM Lf; blank samples did not contain Lf. In order to estimate the effect of cell signal modulators samples were incubated either in the presence or in the absence of the agents.

Lactate content of the erythrocytes

After centrifugation for 10 min at 2,000 \times g erythrocytes were resuspended in 0.4 ml 10% trichloroacetic acid (TCA). For entire precipitation samples were cooled on ice 10 min and then centrifuged again at the same conditions. 0.1 ml from the supernatant was used further according to the prescription of the test kit obtained from Sigma Diagnostics (St. Louis, MO, USA). The method was based on pyruvate reduction in the presence of NADH and lactate dehydrogenase. To estimate lactate, the reaction was carried out in the excess of NAD⁺, and the absorbance of NADH was measured at 340 nm wavelength. The samples for estimation of the Lf stimulatory effect in the presence of test agent contained 50 nM Lf (control) or 50 nM Lf together with the test agent. For the estimation of the flavonoid effect on the lactate generation only agent without Lf was used. The blank samples contained neither agent nor Lf.

Na⁺/K⁺-ATPase activity of the erythrocyte membranes

Samples (50 μ l) were added to 450 μ l of the following solution (per litre): 100 mmol NaCl, 20 mmol KCl, 2.5 mmol MgCl₂, 0.5 mmol EGTA, 50 mmol Tris [tris (hydroxymethyl) aminomethane]-hydrochloride buffer, pH 7.4, 1.0 mmol ATP, 1.0 mmol phosphoenolpyruvate, 0.16 mmol NADH, 5 kU pyruvate kinase, 12 kU lactate dehydrogenase (all from Sigma). After 5 min, 5 μ l of 10 mmol/l ouabain were added to inhibit the ouabain-sensitive ATPase activity. The change in absorbance was monitored at 340 nm (reference wavelength 415 nm) by a twin test (*i.e.*, combination of two assays in one cuvette); rate A (*i.e.*, slope of total ATPase activity), 80–280 s; rate B (*i.e.*, slope of ouabain-resistant ATPase activity), 400–600 s. The difference between the two slopes was proportional to the Na⁺/K⁺-ATPase activity. The samples for the estimation of the Lf stimulatory effect in the presence of test agent contained 50 nM Lf (control) or 50 nM Lf together with the test agent. For the estimation of the flavonoid effect on Na⁺/K⁺-ATPase activity only agent without Lf was used. The blank samples did not contain neither agent nor Lf (Vásárhelyi *et al.*, 1997).

⁵⁹FeLf binding with erythrocyte membranes

Lactoferrin from human milk (Sigma-Aldrich Chemie, Germany) was dialyzed against 0.2 M sodium citrate buffer, pH 4.0, for 24 h at room temperature to remove bound iron. Iron saturation was carried out by adding $1.04 \cdot 10^{-7}$ mol ⁵⁹Fe citrate (Du Pont de Nemours, Mechelen, Belgium) or $1.04 \cdot 10^{-7}$ mol nonlabelled FeCl₃ (Merck, Darmstadt, Germany) to $2.6 \cdot 10^{-8}$ mol Lf (apo-Lf solution) in a final volume of 2 ml. Nonlabelled Lf was used as competitor in the iron-binding experiments. The pH value was adjusted to 7.4. with 0.2 M sodium bicarbonate as described elsewhere (Taleva *et al.*, 1999). The mixture was stirred over-

night at 37 °C, and then dialyzed against 0.02 M sodium bicarbonate and purified through a Sephadex G 75 column to remove the unbound ⁵⁹Fe. Iron saturation was determined twice: by absorbance at 465 nm using $E_{1\%}^{1\text{cm}}(465) = 0.58$ (Taleva *et al.*, 1999), and by calculation according to a ⁵⁹Fe-radioactivity standard line after measuring the radioactivity by a Rack Gamma II 127 counter (Pharmacia LKB, Turku, Finland). Lf used in the experiments was 98% saturated with Fe. The effect of the electronic acceptors was measured after adding 30 and 60 mM ascorbic acid, 2 mM NAD⁺ or FAD to the samples to determine the total and specific binding.

Results

Lf (50 nM) enhanced both the lactate generation and ATPase activity with by 47%, $p < 0.001$. The lactate content in the erythrocytes in the absence of agents was $(5.15 \pm 0.19) \mu\text{mol/g Hb}$, and in the presence of Lf it was $(7.56 \pm 1.21) \mu\text{mol/g Hb}$. The Na-pump activity, when Lf was not presented in the media, was $(4.44 \pm 0.98) \text{U/g protein}$, and in the presence of 50 nM Lf it was $(6.54 \pm 0.90) \text{U/g protein}$ (Table I).

Quercetin and piceatannol had an additive effect on the stimulation of the Na⁺/K⁺-ATPase with Lf (Table I). None of the estimated flavonoids had an effect on the Lf stimulation of the lactate production (Table I). The lactate generation increased when flavonoids and Lf were introduced simultaneously (Table II), but there was no significant difference from the stimulation caused by Lf alone (Table I).

Quercetin enhanced the ATPase activity by 31% ($p < 0.02$), but it did not show an effect on the lactate formation (Table II). Apigenin enhanced reliably (with 50%, $p < 0.001$) the lactate content, but it did not show an effect on the ATPase activity (Table II). Piceatannol did not have any reliable effect on the lactate content and on the Na⁺/K⁺-ATPase activity (Table II).

Agent	Na ⁺ /K ⁺ -ATPase [U/g protein] ($n = 6$)	Lactate [$\mu\text{mol/g Hb}$] ($n = 6$)
50 nM Lf	6.54 ± 0.90	7.56 ± 1.21
1.5 μM Quercetin + 50 nM Lf	$8.50 \pm 1.10^{**}$	7.74 ± 0.25
30 μM Piceatannol + 50 nM Lf	$8.02 \pm 0.85^*$	7.91 ± 0.35
1.5 μM Apigenin + 50 nM Lf	7.28 ± 0.74	7.74 ± 1.17

Table I. Effect of Lf on Na⁺/K⁺-ATPase activity and lactate generation in the presence of flavonoids.

* $p < 0.02$; ** $p < 0.01$.

Agent	Na ⁺ /K ⁺ -ATPase [U/g protein] (n = 6)	Lactate [μmol/g Hb] (n = 6)
Without agents (control)	4.44 ± 0.98	5.15 ± 0.19
1.5 μM Quercetin	5.79 ± 1.39**	5.15 ± 0.81
1.5 μM Quercetin + 50 nM Lf	8.50 ± 1.10*	7.71 ± 0.25*
30 μM Piceatannol	5.08 ± 1.61	5.62 ± 1.14
30 μM Piceatannol + 50 nM Lf	8.02 ± 0.85*	7.91 ± 0.25*
1.5 μM Apigenin	4.44 ± 0.74	7.74 ± 1.17*
1.5 μM Apigenin + 50 nM Lf	7.28 ± 0.74***	7.86 ± 1.75*

* $p < 0.001$; ** $p < 0.02$; *** $p < 0.01$.

Table II. Effect of flavonoids on Na⁺/K⁺-ATPase activity and lactate generation.

Agent	Bound ⁵⁹ FeLf [ng ⁵⁹ FeLf/10 ⁶ cells] (n = 6)	Inhibition (%)
50 nM Lf	62.31 ± 6.33	–
50 nM Lf + 30 mM ascorbic acid	49.98 ± 15.93	20
50 nM Lf + 60 mM ascorbic acid	34.57 ± 6.97 *	44
2 mM NAD ⁺	19.22 ± 6.72 *	68
2 mM FAD	16.99 ± 5.72 *	73

* $p < 0.001$.

Table III. Inhibiting effect of ascorbic acid, NAD⁺ and FAD on ⁵⁹FeLf binding to the erythrocytes.

The simultaneous introduction of ascorbic acid and Lf stimulated reliably the enzyme activity by 39%, $p < 0.01$ [(6.18 ± 1.10) U/g protein], in comparison with the control [(4.44 ± 0.98) U/g protein] (Table II). This effect due to Lf was (6.54 ± 0.90) U/g protein, which stimulated the ATPase at the same level when used alone, and in combination with ascorbic acid it was (6.18 ± 1.10). Ascorbic acid had no effect on the ATPase activity: (4.44 ± 0.98) U/g protein (control without ascorbic acid) and (5.18 ± 0.96) U/g protein (with ascorbic acid), $p > 0.1$.

Ascorbic acid (30 and 60 mM) decreased the specific binding of Lf by 20% ($p > 0.1$) and 44% ($p < 0.001$), respectively. NAD⁺ (2 mM) and FAD (2 mM) also exerted an inhibitory effect on the specific binding (68% and 73%, respectively, $p < 0.001$) (Table III).

Discussion

Hirano *et al.* (1989) showed that flavonoids at concentrations from 40 to 1000 μM inhibit Na⁺/K⁺-ATPase from dog kidney cortex. The background in the literature is that flavonoids exert specific and nontoxic effects at low concentrations and that the higher concentrations have prooxidative and cytotoxic effects. Davis *et al.* (1983) found that quercetin at low concentration (1–10 μM) stimu-

lates the erythrocyte Ca²⁺-ATPase activity and at 50 μM inhibits the enzyme. The plasma concentration of total polyphenol metabolites ranged from 0 to 4 μM (Manach *et al.*, 2005).

Quercetin and piceatannol enhanced the stimulatory effect of Lf on Na⁺/K⁺-ATPase (Table I) and exerted probably similar regulation of the enzyme activity. Quercetin as a redox system is part of the electron transport chain, reducing extracellular ferric cyanide and at physiological conditions is an intercellular substrate of a transplasma oxidoreductase (Fiorani *et al.*, 2002). Some data exist about the participation of Lf in such a chain (Sun *et al.*, 1991). Donating electrons are necessary for the reduction of Fe³⁺ to Fe²⁺ during Lf binding with membrane receptors (Aisen and Brown, 1975). Flavonoids could facilitate the Lf binding because of their iron ion-reducing ability (Middleton *et al.*, 2000). Apigenin is not a participant in the electron transport chain (Schipfer *et al.*, 1985) and has no effect on the Lf stimulation of the pump (Table I).

Our results revealed a stimulating effect of quercetin on the erythrocyte Na⁺/K⁺-ATPase (Table II). In support of these data are the other favourable effects of the intercellular flavonoids pool in the erythrocyte (Fiorani *et al.*, 2002). The used mechanism(s) of quercetin for the stimula-

tion of Na^+/K^+ -ATPase might concern its antioxidative properties (Gao *et al.*, 1997) and also some involvement(s) in the erythrocyte signaling. It was shown an inhibitory effect of quercetin on PKC (Zhang *et al.*, 2004), a negative regulator of the pump activity (Xia *et al.*, 1995). The stimulation of Na^+/K^+ -ATPase was found only for quercetin, but not for apigenin and the resveratrol analogue piceatannol (Table II) and probably assigns enhanced antioxidative properties of quercetin (Gao *et al.*, 1997) or may concern some structural difference between the tested flavonoids.

The erythrocyte glycolytic enzymes, forming complexes with band 3, are regulated by processes of phosphorylation and oxidation (Campanella *et al.*, 2005), while pyruvate kinase (PK) is regulated by phosphorylation/dephosphorylation (Kimer and Westhead, 1979). The band 3 phosphorylation activates the glycolytic enzymes (Campanella *et al.*, 2005).

Apigenin significantly stimulates glycolysis by about 50% (Table II). There are no data that apigenin modulates band 3 phosphorylation, and also apigenin does not participate in the electron transport as a redox system, activating glycolysis (Fiorani *et al.*, 2002). Probably apigenin participates in the regulation of glycolysis as a regulator of PK, using the signal pathways established in other cell types and inhibiting the activity of the cAMP-dependent pathway (Landolfi *et al.*, 1984) or casein kinase 2 (Kuronuma *et al.*, 2004). The stimulatory effect of apigenin on glycolysis (Table II) in the erythrocyte might be indirect, as a consequence of its stimulatory effect on the MAPK pathway enhancing Na^+/H^+ -antiport (Sartoli *et al.*, 1999). It is known that one of the mechanisms for the stimulation of glycolysis is activation of the proton export by the antiport (Madshus, 1988). The stimulatory effect also could be referred to the apigenin ability to maintain the erythrocyte K^+ (Mardonneau-Parini *et al.*, 1986).

An additive effect of apigenin and Lf as stimulators of glycolysis might be expected, but such effect was not observed in their combined introduction (Table II). It is an interesting fact that both modulators have one and the same target – casein kinase 2. Apigenin is an inhibitor (Kuronuma *et al.*, 2004), and Lf is a stimulator of that enzyme

(Mackawa *et al.*, 2002). If Lf uses a signal pathway with casein kinase 2, apigenin might interfere with it as a negative regulator.

Quercetin and piceatannol take part in the regulation of the phosphorylation of band 3 in the presence of the physiological oxidant peroxynitrite (ONOO^\bullet). Quercetin decreases the Syk activity and particularly prevents a phosphotyrosine phosphatase inhibition caused by the free radical peroxynitrite. Resveratrol (the analogue of which is piceatannol) has another mechanism of action; it amplifies the peroxynitrite-dependent upregulation of band 3 tyrosine phosphorylation through the activation of lyn, a kinase of the src family (Maccaglia *et al.*, 2003).

We studied the effect of flavonic compounds on lactate generation without simulation of the model of oxidative stress. In our experiments quercetin and piceatannol did not influence reliably the lactate generation (Table II). It might be supposed that they have opposite and neutralizing each other effect: as inhibitors of tyrosine kinase Syk with target band 3 (Seow *et al.*, 2002) both flavonoids could inhibit glycolysis and, as a part of the electron transport chain, they would drill a stimulatory effect (Fiorani *et al.*, 2002).

The present study showed that ascorbic acid does not have any effect on the activity of the Na^+/K^+ -ATPase and does not change and obstruct the stimulatory effect of Lf on it. But our results showed also that ascorbic acid reliably inhibits the Lf binding to its receptors (Table III). It might be supposed that this inhibitory effect concerns the competition for electrons, necessary for the Lf binding to the receptor. Ascorbic acid may use these electrons for maintaining its own reduced state. Erythrocyte hemolysates showed both NAD- and NADPH-dependent ascorbate radical reduction. The latter was partially due to thioredoxin reductase (May *et al.*, 2004). Probably this enzyme competes with Lf for electrons and thus complicates its binding to the receptor. Lf, as an antioxidant, probably neutralizes the pro-oxidative properties of higher doses of ascorbic acid. Because of the decrease of Lf binding in the presence of NAD^+ and FAD (Table III), it is possible that an unknown redox process(es) with the participation of the membrane oxidoreductase(s) might be involved.

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