

Lactoferrin-Protector against Oxidative Stress and Regulator of Glycolysis in Human Erythrocytes

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Binding of lactoferrin (Lf) to its membrane receptors requires an electron for the reduction of Fe^{3+}LF to Fe^{2+}LF . It is possible that glyceraldehyde –3-phosphate dehydrogenase, a glycolytic enzyme part of the erythrocyte membrane, delivers that electron. Then Lf, obtaining an electron from the coenzyme NADH, might stimulate glycolysis, which requires the oxidised state of the coenzyme NAD^+ . Such possibility is supported by the finding that another extracellular e^- acceptor – potassium ferricyanide activates glycolysis by the similar mechanism. Present results show that ferricyanide inhibited the specific ^{59}Fe -lactoferrin binding to its erythrocyte membrane receptors. It may be assumed that ferricyanide competes with lactoferrin for an electron which leads to decrease of the binding of ^{59}Fe -lactoferrin to its receptors. Lactoferrin (50 and 100 nm), similar to ferricyanide, increased the accumulation of lactate (respectively by 25% and 30%). These results support the assumption that ferricyanide and lactoferrin are final acceptors of a common electron transport chain connected with the regulation of glycolysis.

We established an antioxidative effect of lactoferrin on erythrocytes, which was expressed as: a) an influence on content and on activity of intracellular antioxidants – namely an enhancement of the content of reduced glutathione; b) a decreased content both of products of lipid peroxidation (thiobarbituric acid reactive substances) and hemolysis under normal conditions and oxidative stress.

Lactoferrin is capable to bind metal ions and thus to block their catalytic participation in the oxidative disturbances of the membrane. In most of our experiments there were no metal ions in the incubation mixtures (except those stimulating oxidative stress). Our results showed that Lf limited both the generation of thiobarbituric acid reactive substances and hemolysis in the absence of metal ions in the media, as well as in their presence. These facts suggest that probably the antioxidative property of lactoferrin is glycolysis stimulation, leading to increased formation of ATP, which is necessary to maintain the ion gradient, membrane potential and morphology of the erythrocyte.

Key words: Lactoferrin, Antioxidant, Glycolysis

Introduction

Lactoferrin (Lf) is a metal-binding protein with various biological effects. It acts as a specific transcription factor (Beaule, 1995), activates a transduction pathway with the participation of a mitogen-activated protein kinase (MAP-kinase) in lymphoblast cell line (Duthille *et al.*, 1998). It has an antibacterial (Arnold *et al.*, 1982), antiviral (Hasegawa *et al.*, 1994), antitumor (Bezault *et al.*, 1994), antiatherogenic (Kaijkawa *et al.*, 1994), antioxidative activity (Cohen *et al.*, 1992) and immunoregulatory functions (Manev *et al.*, 1998).

Our previous studies showed the existence of a specific binding sites for Lf on the erythrocyte cell

membrane (Taleva *et al.*, 1999). Lf-receptor interaction probably is the first stage of the effect of Lf on some erythrocyte functions. It might be assumed that the effects of Lf on the functional activity of the erythrocytes are connected with control on the activity of the glycolytic pathway, which is the only supplier of energy for the erythrocytes and the mechanisms for antioxidative protection, maintaining the membrane potential of the red blood cell and the native conformation of hemoglobin (Clemens and Waller, 1987).

It has been reported that some extracellular redox systems, for example potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), stimulate glycolysis in erythrocytes being a final acceptor for the electrons (e^-) gener-

ated by the glyceraldehyde 3-phosphate dehydrogenase reaction (Low *et al.*, 1990). The existence of an erythrocyte transmembrane e^- -transporting system connected with the transferrin receptors was shown experimentally (Orringer and Roer, 1979). That system reduces $K_3Fe(CN)_6$ and removes protons (H^+) from the cell interior towards the cell membrane (Low *et al.*, 1987). Transferrin, as a final physiological acceptor in that chain, probably accepts an e^- , necessary to change over from Fe^{3+} transferrin to Fe^{2+} transferrin while binding to the erythrocyte membrane receptors (Aisen and Brown, 1975; Aisen and Leibman, 1972). Some data exist about the participation of Lf in such e^- -transporting chain, that also leads to a proton release and an active exchange of H^+ against extracellular Na^+ (Sun *et al.*, 1991). It has not yet been reported whether Lf and/or transferrin stimulate glycolysis as an extracellular redox system similar to $K_3Fe(CN)_6$.

In spite of having an effective antioxidative defence, erythrocytes are susceptible to oxidative stress. They contain high amounts polyunsaturated fatty acids, molecular oxygen and ferrous ions in a ligand state. Cell injury provoked by lipid peroxidation of cell membranes can range from increased permeability to cell lysis (Clemens and Waller, 1987). Binding of Lf to the erythrocytes could limit the processes of membrane lipid peroxidation, because Lf is not entirely saturated and is capable additionally to bind iron ions (Ainscough *et al.*, 1979), that are cytotoxic activators of the lipid peroxidation (Clemens and Waller, 1987). Lf, as a final acceptor for the e^- carried by the transmembrane electron transporting chain (Sun *et al.*, 1991), probably could also influence the state of the intracellular antioxidative protection of the red cell.

The aim of the present study was to assess if Lf, similar to potassium ferricyanide, could act as an extracellular electron acceptor and stimulate glycolysis, to maintain the intracellular antioxidative protection of the erythrocytes and to limit the cell injury either in normal conditions or in simulated oxidative stress.

Materials and Methods

Isolation of erythrocytes

Heparinized fresh blood from healthy donors was centrifuged at $2000 \times g$ for 5 min at $4^\circ C$ and

the pellet was resuspended in 4 volumes phosphate buffered saline (PBS) pH 7.4. After three times washing at 1800, 1500, $1300 \times g$ the erythrocytes were isolated by density separation (Cohen *et al.*, 1976). The erythrocyte fraction was resuspended in PBS pH 7.4 to obtain a cell concentration $2 \times 10^7/ml$, counted in Burker's camera by a Standard KF2 microscope (Carl Zeiss, Jena, Germany). The suspension did not contain other cell species.

^{59}Fe -Lf and $K_3Fe(CN)_6$ as electron acceptors;

^{59}Fe labelling of Lf

Lactoferrin from human milk (Sigma-Aldrich Chemie, Germany) was dialysed 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 of 1.04×10^{-7} mol ^{59}Fe -citrate (Dupont de Nemours, Belgium) or 1.04×10^{-7} mol nonlabelled $FeCl_3$ (Merck, KGaA, Darmstadt, Germany) to 2.6×10^{-8} mol Lf (2 ml apo-Lf solution) in final volume of 2 ml. The non-labelled Lf was used as competitor in the iron-binding experiments. pH was adjusted to 7.4 by 0.2 M sodium bicarbonate solution as described elsewhere (Maneva *et al.*, 1983). The mixture was stirred overnight at $37^\circ C$ and then dialysed 2 h against 0.02 M sodium bicarbonate and purified through a Sephadex G 75 column to remove the unbound ^{59}Fe . Iron saturation was determined twice: by absorbance at 465 nm using $E^{1\%}_{465} = 0.58$ (Cox *et al.*, 1989), and by calculation according to a ^{59}Fe -radioactivity standard line after measuring radioactivity by Rack Gamma II 127 counter (Pharmacia LKB, Turku, Finland). Lf used in the experiments was 98% saturated with Fe.

^{59}Fe -Lf binding experiments

Samples were performed in quadruplicates and the tubes for measuring the nonspecific binding contained: 0.01–0.1 ml ^{59}Fe Lf (0.13 – 1.3×10^{-11} mol), 0.05 ml erythrocyte cell suspension (1×10^6 erythrocytes), 1.3×10^{-9} mol (100 fold excess) human non-labelled FeLf and PBS pH 7.4 up to the total volume 0.4 ml. Total binding was measured in the absence of unlabelled FeLf, substituted by the same volume PBS. Binding experiments were carried out at $37^\circ C$. After 30 min incubation the reaction was stopped by addition

of 1 ml ice-cooled PBS and cells collected by centrifugation at 4 °C for 10 min at $6000 \times g$. Radioactivity bound to the sedimented erythrocytes was measured and calculated as nmol Lf. Specific binding was calculated as a difference between the total and the nonspecific binding (Maneva *et al.*, 1983; Maneva *et al.*, 1993). As an electron acceptor 2 mM potassium ferricyanide was used.

The hemoglobin concentration of the erythrocyte suspension containing 20×10^6 cells/ml was determined according to Beutler (1975a).

The effect of 10% iron saturated Lf on the studied parameters was assessed using various concentrations of the protein: lower than the normal serum concentrations, normal and higher than the normal. According to the different authors, normal serum levels of Lf vary from 6–1740 ng/ml (37.5 nM–8.7 μM). In pathological situations – infections, blood malignant diseases and hormonal disturbances, these values enhance repeatedly (Levay and Viljoren, 1995). *In vivo* human Lf is about 4% to 44% saturated with iron (Aisen and Leibman, 1972).

Lactate and glutathione content of the erythrocytes

Lactate content in the erythrocytes was measured by a test kit reagent obtained from “SIGMA Diagnostics” (St. Louis, USA). The method was based on the reaction of pyruvate oxidation in the presence of NADH and lactate dehydrogenase. To estimate lactate, the reaction was carried out from left with excess NAD⁺ and NADH was measured. 50 μl erythrocyte suspension (2×10^7 cells per ml) was incubated 30 min at 25 °C. Each sample contained 20–100 nM Lf. The blank samples did not contain Lf. After centrifugation for 10 min at $2000 \times g$ 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 enclosed in the kit.

Measuring of the glutathione (G-SH) content in the erythrocytes was carried out according to the method of Beutler (1975b), based on the ability of the dye 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The products with pale yellow color have an absorption maximum at 412 nm. 50 μl erythrocyte suspension (20×10^6 cells per ml)

was incubated for 1 h with various concentrations of Lf (Table II). After the incubation samples were centrifuged 10 min at $2000 \times g$ and resuspended in 0.3 ml double distilled water. To the prepared lysate was added 0.75 ml buffer for precipitation: 0.17 M *meta*-phosphoric acid (glacial), 0.068 M Na₂EDTA, 5.1 M NaCl. After 5 min at 18 °C and another centrifugation for 10 min at $2000 \times g$ 0.4 ml of the supernatant were mixed with 1.6 ml Na₂HPO₄ (0.3 M) and 0.2 ml DTNB (0.5 mM DTNB in 39 mM trisodium citrate solution). The absorbance at 412 nm was read against blank sample containing only buffer for precipitation.

Incubation of the erythrocytes in simulated conditions of oxidative stress

Our system contained 0.2 mM ascorbic acid + 10 mM FeSO₄, which was near to that used by Fukuzawa *et al.* (1981). 8×10^9 cells were mixed with 50 and 100 nM Lf (50 or 100 μl, respectively), 20 ml 10^{-5} M FeSO₄, 40 ml 10^{-4} M ascorbic acid and PBS (pH 7.4) to a total volume 2.5 ml. The control samples did not contain Lf. After a 24 h incubation at 4 °C thiobarbituric acid reactive substances (TBARS) and degree of hemolysis were assayed.

Thiobarbituric acid reactive substances (TBARS)

After a 24 h incubation at 4 °C either in the presence of 160 nM Lf or in the absence of Lf, the samples containing 8×10^9 cells were centrifuged for 10 min at $2000 \times g$ and resuspended in 1.5 ml 10% TCA. After another centrifugation 10 min at $4000 \times g$, 0.6 ml from the supernatant were diluted with 0.8 ml 0.67% thiobarbituric acid (TBA). Tubes were placed in water and boiled for 60 min. After cooling absorbance at 635 nm was measured against water. Results were calculated by a standard curve and presented as μmol malonyl dialdehyde (MDA)/ 8×10^9 erythrocytes (Mengel and Kann, 1966).

Hemolysis

Hemolysis was measured according to Cardenas *et al.* (1992). The optical density of Hb, released from the sedimented erythrocytes (8×10^9 in each tube), was used as a criteria for the degree of hemolysis. The extinction coefficient $E_{412} =$

127 mm · cm⁻¹, corresponding to 1 mM Hb (Cardenas *et al.*, 1992) was used for the calculation of the Hb concentration.

The degree of hemolysis was determined after a 24 h incubation at 4 °C: a) without oxidative stress and without Lf; b) without oxidative stress in the presence of Lf (6.25–100 nM); c) with oxidative stress solutions without Lf; d) with oxidative stress solutions and in the presence of Lf (25 and 50 nM).

Results

In the absence of K₃Fe(CN)₆ the erythrocytes bound specifically 62,31 ± 6,33 nmol Lf, and in the presence of K₃Fe(CN)₆–49.95 ± 4.36 nmol Lf. K₃Fe(CN)₆ inhibited with 21% (*p* < 0.05) the ⁵⁹FeLf binding to the membrane receptors on the erythrocyte membrane.

Lf in concentrations 50 to 100 nM enhanced significantly the lactate content in the erythrocytes – from 30% to 41% (Table I). A positive correlation was observed between the concentration of the added Lf and the formed lactate: *r* = 0.965, *p* < 0.001, *n* = 5.

Increased concentration of G–SH was measured in the presence of 25–100 nM Lf. The enhancement was statistically reliable only for the higher concentrations: 75 and 100 nM Lf – 0.214 ± 0.070 (*p* < 0.05) and 0.230 ± 0.110 (*p* < 0.025) μmol G–SH/g Hb, respectively (Table II). A positive correlation was found between the Lf concentration in the samples and the values of the reduced glutathione: *r* = 0.991, *p* < 0.001, *n* = 5.

Lf (160 nM) limited the MDA generation in the erythrocytes during their storage in normal conditions and in simulated conditions of oxidative stress. The protective effect was found to be stronger in the conditions of oxidative stress:

Table I. Stimulatory effect of lactoferrin on the lactate generation by erythrocytes.

Lactoferrin [nM]	Lactate μmol/g Hb x ± SD (<i>n</i> = 8)	% Increase vs. control	<i>p</i>
0	1.38 ± 0.25	–	–
25	1.50 ± 0.35	9	> 0.1
50	1.80 ± 0.41	30	< 0.025
75	1.90 ± 0.45	38	< 0.001
100	1.95 ± 0.49	41	< 0.001

Table II. Stimulatory effect of lactoferrin on the content of reduced glutathione.

Lf [nM]	G SH μmol/g Hb x ± SD (<i>n</i> = 24)	% Increase vs. control	<i>p</i>
0	0.166 ± 0,090	–	–
25	0.187 ± 0,075	12	> 0.1
50	0.205 ± 0,100	19	> 0.1
75	0.214 ± 0,070	31	< 0.05
100	0.230 ± 0,110	28	< 0.025

In normal conditions the MDA content was 8.0 ± 1.06 μmol/8 × 10⁹ erythrocytes and in the presence of Lf – 6.56 ± 0.61 μmol MDA/8 × 10⁹ erythrocytes (*p* < 0.001). Lf decreased the MDA content with about 18% (Fig. 1).

In oxidative stress the MDA content was 12.48 ± 0.75 μmol/8 × 10⁹ erythrocytes and in the presence of Lf – 7.99 ± 0.48 μmol MDA/8 × 10⁹ erythrocytes (*p* < 0.001). Lf decreased the MDA content with 36%, *p* < 0.001 (Fig. 1).

Lf limited the spontaneous hemolysis as well as the hemolysis in oxidative stress conditions (Fig. 2).

Lf (6.25 to 50 nM) decreased reliably the spontaneous hemolysis and the effect did not depend proportionally from the used concentration: In normal conditions the spontaneous hemolysis was 1550 ± 897 μM Hb/8 × 10⁹ erythrocytes. Maximal

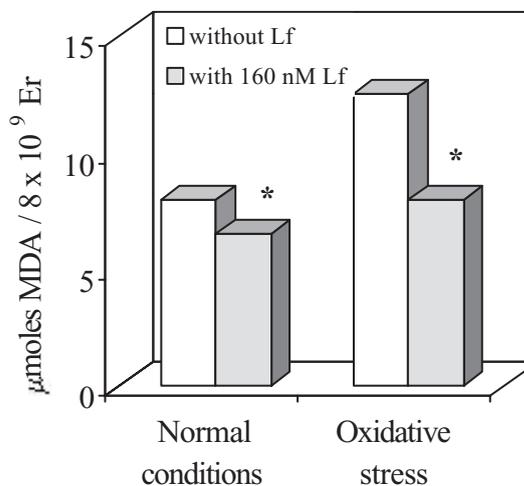


Fig. 1. Inhibition of the thiobarbituric acid reactive substances (TBARS) generation by lactoferrin (Lf) * *p* < 0.001. MDA, malonyl dialdehyde; Er, erythrocytes.

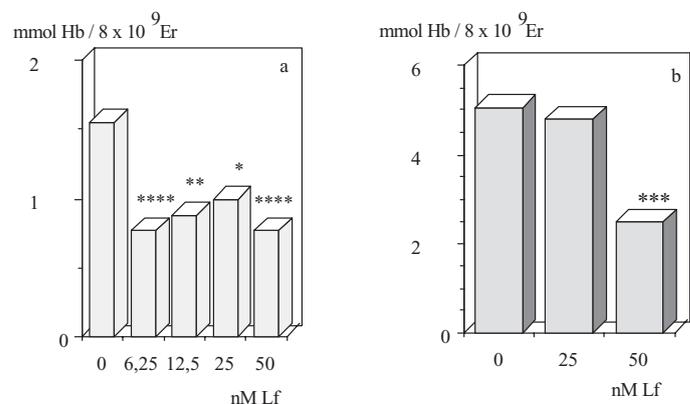


Fig. 2. Inhibition of the hemolysis by lactoferrin (Lf): a) in normal conditions ; b) in oxidative stress. * $p < 0.05$, ** $p < 0.025$, *** $p < 0.02$, **** $p < 0.01$. Er, erythrocytes; Hb, hemoglobin.

inhibitory effect was observed when the highest and the lowest concentrations were used: with 6.25 nM Lf was $770 \pm 370 \mu\text{M Hb}/8 \times 10^9$ erythrocytes, and with 50 nM Lf – $770 \pm 378 \mu\text{M Hb}/8 \times 10^9$ erythrocytes respectively (Fig. 2a).

Lf (25 and 50 nM) inhibited the hemolysis in oxidative stress conditions. The lower concentration (25 nM) negligibly decreased the degree of hemolysis. The effect was significant only for the higher concentration (50 nM Lf), where the released Hb was about two-fold less, compared with the samples without Lf: $2478 \pm 1149 \mu\text{mol Hb}/8 \times 10^9$ erythrocytes and $5035 \pm 1150 \mu\text{mol Hb}/8 \times 10^9$ erythrocytes respectively ($p < 0.02$), (Fig. 2b).

Discussion

It has been reported that potassium ferricyanide stimulates glycolysis in erythrocytes (Mishra and Passow, 1969) being a final acceptor for the electrons, generated by the glyceraldehyde-3-phosphate dehydrogenase reaction (Low *et al.*, 1990). Experimentally was shown the existence of an erythrocyte transmembrane electron-transporting system connected with the transferrin receptors (Orringer and Roer, 1979), that reduces $\text{K}_3\text{Fe}(\text{CN})_6$ and removes protons from the cell interior towards the cell membrane (Low *et al.*, 1987). It was suggested that ferricyanide and transferrin participate in a common e^- transporting system and *in vivo* transferrin is the final acceptor (Low *et al.*, 1987).

In spite of some differences in the chemical properties and the functions of the serum transferrin and Lf (Aisen and Leibman, 1972), probably

Lf has the same property to be an extracellular e^- acceptor as transferrin: 1) No data have been reported yet about any presence of Lf inside red blood cells (Yamada *et al.*, 1987); 2) Both Lf and serum transferrin act as an e^- acceptors by one the same mechanism while bind to their membrane receptors. During that process the iron carried by them changes over from Fe^{3+} to Fe^{2+} (Aisen and Braun, 1975); 3) Lf, similarly to transferrin (Goldenberg *et al.*, 1990), as a final acceptor for e^- , can participate in a chain of oxidoreductive reactions at plasma membrane, leading to the activation of Na^+/H^+ -exchange (Sun *et al.*, 1991). Present results show decrease in the ^{59}Fe -Lf binding to its receptors at the erythrocyte plasma membrane in the presence of ferricyanide. The inhibitory effect might due to a competition for one and the same e^- between ferricyanide and Lf. Similar to ferricyanide (Low *et al.*, 1990), Lf increased the accumulation of lactate, a final product of glycolysis (Table I). These results support the suggestion that ferricyanide and Lf are final acceptors of a common e^- transport chain, connected with the regulation of glycolysis.

Erythrocyte membrane is susceptible to oxidants and is stabilised by antioxidants (Clemens and Waller, 1987). Present results show an antioxidative effect of Lf on erythrocytes, which was expressed as an enhancement of the reduced glutathione content (Table II), a decreased content of TBARS (Fig. 1) and a limited hemolysis (Fig. 2). Lf in plasma and body fluids is partially saturated with iron and drills an antioxidative effect by binding additionally iron (Fe^{2+}), that catalyses hydroxyl radical formation in a non-catalytic (Fe^{3+})

form (Clemens and Waller, 1987). In most of our experiments there were no metal ions in the incubation mixture (except those, simulating oxidative stress). Our results showed that Lf limited the TBARS generation in the absence of metal ions, as well as in their presence (Fig. 1). Lf also decreased the degree of hemolysis in conditions without oxidative stress. These facts suggest that the favourable effects of Lf on the studied parameters due to an integration of its metal-binding property, influence of glycolysis, ion transport and/or membrane phosphorilation:

1) The stimulation of glycolysis (Table I) leads to an increased generation of ATP, necessary for maintaining the ion gradient, membrane potential and morphology of erythrocytes. Maintaining of the membrane potential, Lf could limit the oxidative changes (Scott and Rabito, 1988) and thus to decrease the necessity of reduced glutathione. Our results shown lower content of TBARS and degree of hemolysis (Fig. 1 and 2) and higher level of reduced glutathione in the presence of Lf (Table II).

2) Lf can participate in oxido-reductive reactions at the cell membrane, which lead to activation of the Na^+/H^+ exchange (Sun *et al.* 1991). So Lf might participate in maintaining of optimal ion gradient.

Activation of Na^+/H^+ exchange enhances the intracellular pH. It is well known that as pH increases within the physiological limits, the metabo-

lite activity of the cell also increases (Madshus, 1988). This mechanism is responsible for the stimulatory effect of Lf on the growth of K562 cell lines (Sun *et al.*, 1991). Similar mechanism might activate the synthesis of reduced glutathione and/or NADPH, which maintains the level of reduced glutathione in the erythrocytes.

3) It was found that ferricyanide initiates signal transduction mediated by protein tyrosine kinase, which phosphorylates the glycolytic enzyme's binding site on the erythrocyte membrane band 3 (Boivin, 1988). That phosphorylation leads to enzyme displacement and activation (Low *et al.*, 1990). Similar mechanism of Lf participation in the signal transduction might be discussed, more than it has been reported that Lf stimulates mitogen activated protein kinase (MAPK) in human lymphoblast cell line (Duthille *et al.*, 1998) and neutrophils (Oh *et al.*, 2001).

This study presents Lf as a regulator of the metabolic activity in erythrocytes, concerning glycolysis and antioxidative status. These effects probably are mediated by the Lf-receptor interaction (Taleva *et al.*, 1999): Lf, as a final membrane acceptor of an electron transport chain, might stimulate glycolysis by the restoring the NAD^+ level. Fe^{3+}Lf accepts an e^- from NADH, generated by glyceraldehyde-3-phosphate dehydrogenase reaction. This e^- reduces Fe^{3+}Lf to Fe^{2+}Lf , and that reduction is an essential stage of Lf-receptor interaction.

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