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EFFECT OF SOME ENZYMES AND INHIBITORS ON THE LACTOFERRIN BINDING TO ERYTHROCYTE MEMBRANE RECEPTORS

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ABSTRACT

Lactoferrin (LF) is an iron-binding glycoprotein and a regulator of cell function with a wide range of biological activities. In our previous studies we reported Lf receptors on erythrocytes. The structure of erythrocyte membrane receptor for Lf is still unknown. The aim of the study is to evaluate the specific binding of Lf to the erythrocytes' membrane receptors in the presence of some enzymes, atractyloside (inhibitor of nucleotide transport) and N-ethylmaleimide (inhibitor of -SH groups). **METHODS**: Erythrocytes were isolated from heparinized fresh blood from healthy donors by density fractionation (Cohen et al.1976). Specific binding was determined according to Maneva et al. (1983). **RESULTS:** Pepsin increased specific binding of Lf to membrane receptors. Proteinase K, pronase E, β-galactosidase, α-glucosidase, neuraminidase, N-ethylmaleimide and atractyloside reduced the specific binding. Lack of specific binding was found in the presence of trypsin, lactoperoxidase and DNA-ase. **CONCLUSION**: Our results suggest the importance of specific amino acids, glycosidic bonds, and the SH-groups in the LF-receptor interaction. The likelihood of some electrostatic interactions of LF with negatively charged molecules, as well and its ability to form complexes with other proteins should be also considered for evaluation of Lf-receptor interactions.

Key words: lactoferrin, receptors, enzymes, inhibitors, erythrocytes

INTRODUCTION

Lactoferrin (Lf) is a metal-binding glycoprotein (Spik et al. 1994) and a transcription factor (Fleet and Mayer, 1995) with antioxidative (Cohen et al., 992), anti-inflammatory, immunomodulatory (Legrand, 2012), anti-cancerogenic (Furlong et al, 2006) anti-bacterial (Weinberg, 2007), anti-viral (Superti et al. 2001), anti-atherogenic (Ji et al. 1994), and anti-thrombotic properties (Levy-Toledano et al., 1995).

Lf effects are mediated by its binding to the membrane receptors. The LF receptors on lymphocytes membrane are known to be glycoproteins but they are still not very well defined. LF receptors are found to be monomers, dimers and trimers with a M.W. from 42 to 110 kDa. The binding affinity ranges from 10^5 M^{-1} to 10^{10} M^{-1} depending on the species and the cell type (Maneva et al.1983; Iyer et al.1994; Eda et al. 1997; Furlong et al. 2006; Legrand et al. 1997).

Our previous studies demonstrated the presence of Lf receptors on the erythrocyte membrane (Taleva et al., 1999). Lf-receptor binding results in stimulation of glycolysis, antioxidative protection (Maneva et al., 2003) and Na^+/K^+ - ATPase activity (Maneva et al., 2007). Lf–receptor interaction may result in short–term effects of regulation, including changes in association, phosphorylation and oxidation of the membrane proteins. The structure of erythrocyte membrane receptor for Lf is still unknown.

The aim of the recent investigation is to evaluate the specific binding of Lf to the erythrocyte membrane receptors in the presence of some enzymes, atractyloside (inhibitor of nucleotide

transport) and N-ethylmaleimide (inhibitor of -SH groups).

MATERIALS AND METHODS

Human Lf was isolated from human milk according to Maneva et al. (1983).

Pepsin, trypsin, proteinase K, pronase E, β-galactosidase, α-glucosidase, neuraminidase and DNA-ase, atractyloside and N-ethylmaleinide were purchased from "Sigma" (USA).

Apo-Lf and ^{59}Fe Lf: Human Lf was dissolved in double distilled water and dialyzed against 0.2M sodium citrate buffer (pH 4.0) for 24 h at room temperature for total removal of physiologically bound iron. 26 x 10^{-9} moles Lf were added to 104 x 10^{-9} moles ^{59}Fe citrate and pH adjusted to 7.4 by 0.02 M sodium hydrogen carbonate for removal the unbound ^{59}Fe. ^{59}FeLf was saturated up to 98% with iron and SDS-PAGE presented only one peak of radioactivity (Maneva et al. 1983).

Isolation of erythrocytes: Heparinized fresh drawn blood from healthy donors was centrifuged at 2000 x *g* for 5 min at 4°C and the cells were resuspended in 4 volumes phosphate buffered saline (PBS), pH 7.4. After three times washing at 1800, 1500, 1300 x *g* the erythrocytes were isolated by density separation (Cohen et al., 1976).

Binding experiments: The enzymes were dissolved *ex tempore* and applied in the following concentrations per sample: trypsin in PBS 7.4-1600 UI, pepsin in sodium acetate buffer pH 7.1 - 1UI, pronase E in Tris-HCL buffer 7.1 - 0.02 UI, β-galactosidase -13 UI, α-glucosidase - 0.75 UI, neuraminidase and DNA-ase - 25 UI all Tris-HCL pH 7.1. Enzymes were added to the samples in total volume of 0.5 ml. N- ethylmaleimide 0.2-0.7 x 10^{-3} M and 10^{-7} - 10^{-3} M atractyloside were used as metabolic inhibitors. Quadruplicates were performed for each sample that contained 1 x 10^{6} erythrocytes in PBS pH 7.4, 0.01-0.1 ml ^{59} FeLf, 0.05 ml erythrocyte suspension. ^{59}FeLf binding to the receptor was performed either in the presence of 1.3 x 10^{-9} moles unlabelled FeLf (nonspecific binding) or in the absence of it (specific binding) (Scatchard, 1949). Binding experiments were carried out at 37°C. After 30 min of incubation the reactions were stopped by addition of 1 ml ice-cold PBS and cells were collected by centrifugation at 4°C for 10 min at 6000xg. Radioactivity bound to the sedimented erythrocytes was measured by Rack Gamma 127 counter (Pharmacia LKB, Sweden) and calculated as moles Lf.

RESULTS

Pepsin increased specific binding up to 23%. Specific binding is reduced reliably by proteinase K (6%), pronase E (10%), β-galactosidase (13%,), β-glucosidase (10%), neuraminidase (9%,). Lack of specific binding was obtained in the presence of trypsin, lactoperoxidase and DNA-ase **(Table 1)**.

Table 1. *Effect of some enzymes of the binding of ^{59}Fe-Lf to erythrocyte membrane receptors*

Enzyme	Total binding (T)	Non-specific binding (B)	Specific binding (% from total binding)[a]
Without enzyme	190.48 ± 4.52	162.32 ± 5.36****	15
Trypsin	205.96 ± 24.53	179.73 ± 29.66	13
Pepsin	223.79 ± 28.80	171.29 ± 9.34**	23.
Proteinase K	173.30 ± 9.77	162.54 ± 3.80*	6
Pronase E	173.01 ± 7.73	156.44 ± 7.10**	10
β -galactosidase	172.20 ± 6.28	150.60 ± 9.12****	13
α-glucosidase	169.69 ± 10.12	153.26 ± 8.69***	10
Neuraminidase	175.86 ± 12.36	159.81 ± 8.32*	9
Lactoperoxidase	149.82 ± 6.82	152.41 ± 13.63	-2
DNA-ase	164.68 ± 17.97	154.14 ± 10.10	6

[a]Total binding = 100% ; p – comparison between T and B; * - $p<0.05$; ** - $p<0.025$; *** $p<0.01$, **** $p<0.001$

N-ethylmaleimide and atractyloside inhibit Lf specific binding up to 39% and 48%, resp. **(Figure 1)**

DISCUSSION

Trypsin cleaves peptide chains mainly at the carbonyl side of the basic amino acid lysine or arginine. Probably, such chemical bonds are essential for LF-receptor interaction (Legrand et al. 1997; El Yazidi-Belkoura et al. 2001) because in the presence of trypsin the specific binding was not found **(Table 1).**

Pepsin is the most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine. Pepsin enhanced almost two times Lf specific binding. The possible reason for this can be eventual conformational changes facilitating Lf binding **(Table 1).**

Proteinase K is commonly used for its broad specificity. Proteinase K is able to digest native keratin (hair), hence, the name "Proteinase K". The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids proteinase K decreased almost two times Lf specific binding possibly destroying important links with the participation of aliphatic or aromatic amino acids **(Table 1)**.

Pronase E is a non-specific protease. Its proteolytic activity extends to both denatured and native proteins. Our results have shown that specific binding is reduced by pronase E (10%, $p<0.01$) **(Table 1).**

β-Glucosidase, α-Glucosidase and Neuraminidase inhibit Lf specific binding to membrane receptors. These enzymes are responsible for breaking specific bonds thus reducing the possibility for specific LF-receptor interaction **(Table 1).** LF and its receptor are glycoproteins (Eda et al.1996). It can be assumed that due to well-established similarity in the carbohydrate component of their molecules, LF and band 3 may share the same binding sites on the monocyte membrane surface. It was found that the presence of LF deteriorates erythrocyte band 3 protein binding to monocytes. Anti - band 3 IgG recognizes sialylated carbohydrate chain of the molecules of the band 3 and the LF (Audo et al., 1996). Our results are consistent with these data since reduced Lf binding in the presence of neuraminidase was found **(Table 1).**

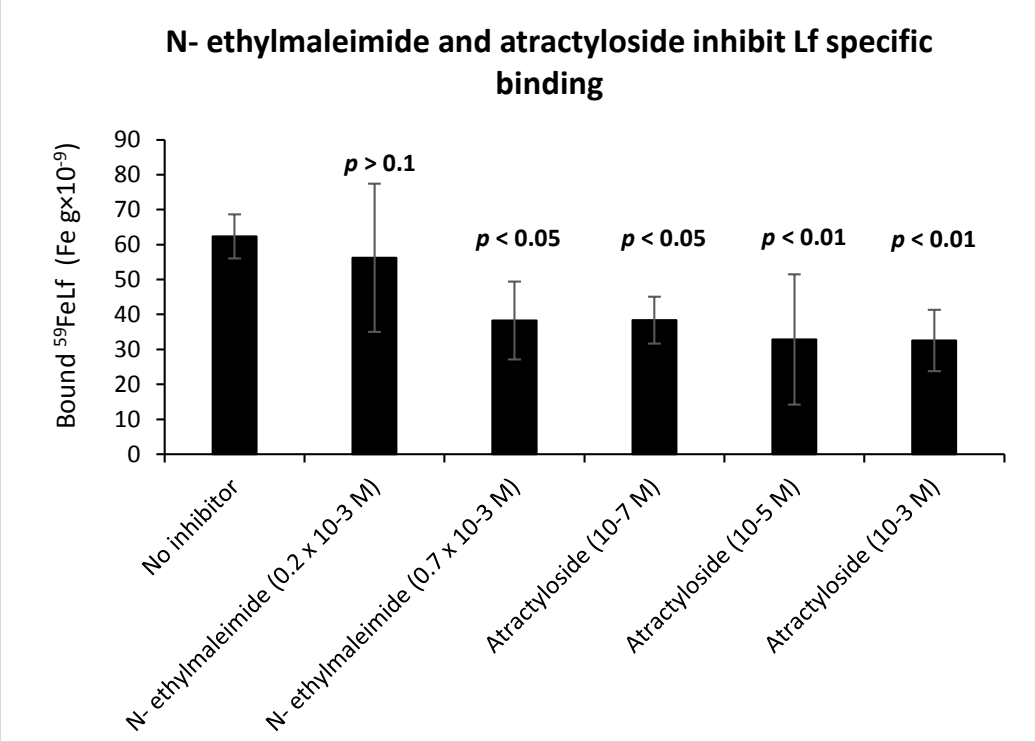

Figure 1

Lactoperoxidase **-** LF forms complexes with milk proteins. It is possible that lactoperoxidase could engage Lf in such a complex (Hekman, 1971), which in turn cannot be "recognized" by the receptor.

DNA-ase **-** Lf binds to cell membrane DNA (Bennett et al. 1983; Bennett et al. 1986). It is considered a protective mechanism through which the foreign genetic material stays restricted on the cell surface and prevents it from entering the cells (Adams, 1985). Most probably the DNA-ases are responsible for destroying LF-DNA-Lf receptor complexes thus abolishing LF binding to its membrane receptors **(Table 1).**

N-etilmaleimide forms covalent bonds with -SH groups and probably blocks the cysteine activity in the receptor molecule. It is likely to assume that thiol groups participate in Lf-receptor interaction **(Figure 1)**. The extracellular sequence of the receptor for transferrin contains 648 amino acids, 8 out of it are cystein (Feelders et al. 1999). Because of the established structural homology between Lf and transferrin, a similar organization of the lactoferrin binding sites could be speculated.

Atractyloside is an inhibitor of nucleotide transport. Its inhibiting effect could interfere with the nucleotide export and erythrocyte bioenergetics. A steric hindrance between transport system and receptor molecule or, a decrease in the specific binding due to disturbance of the energy production could be anticipated **(Figure 1).**

CONCLUSION

Our results suggest the importance of specific amino acids, glycosidic bonds, and the SH-groups in the LF-receptor interaction. The likelihood of some electrostatic interactions of LF with negatively charged molecules, as well and its ability to form complexes with other proteins should be considered also for Lf-receptor interactions' evaluation.

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