Antioxidant Properties of Lactoferrin from Human Milk

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Chemiluminescent methods showed that human lactoferrin more effectively inhibits free radical processes in the Fenton reaction than histidine and mannitol, usual free radical scavengers. Human lactoferrin added to yolk lipoprotein suspension in the presence of rhodamine 6G reduces the intensity of fast flash of Fe^{2+}-induced chemiluminescence by 37%. Complete saturation of lactoferrin with iron reduces its antioxidant properties by 15.4%, the intensity of chemiluminescence being below the control by 25.7%.

Key Words: *lactoferrin; apolactoferrin; hydrogen peroxide; chemiluminescence; free-radical reactions*

Lactoferrin (LF), an important milk constituent, is involved in metabolic reactions, participates in the regulation of cell proliferation and immune response, and possesses important nutritional and antibacterial properties [4,5,9]. The role of LF in the mechanisms of antioxidant protection of breast milk remains poorly understood.

Redox reactions in the organism are an essential part of metabolic processes and contribute to energy supply and transport and utilization of O_2 in tissues. Apart from oxidative phosphorylation utilizing 90% O_2, reactions generating active oxygen metabolites always occur in the organism. Apart from tetravalent reduction of O_2 in the redox reactions, its mono-, di- and trivalent reduction occurs, in which reactive oxygen forms ($O_2^{\bullet-}$, $H_2O_2^{\bullet}$, $OH^{\bullet}$, and $^1O_2$) with high destructive potential are generated [2].

The generation of these radicals is most effectively induced by metals with alternating valency, particularly F^{2+} [3]. Abundant hemin and non-hemin iron compounds are potential centers of radical formation [4,5,9]. LF binds free Fe, which initiates and catalyzes free-radical processes, in particular lipid peroxidation (LPO) in biomembranes [4,5,8-10].

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The aim of the present study was to investigate the effect of LF and the degree of its saturation with iron on the intensity of free radical processes.

MATERIALS AND METHODS

The following chemicals and reagents were used: chromatographic carriers Servacell CN-52 (Reanal) and Toyopearl HW-52 (Toyo-Soda); SDS (Serva); commercial LF and apoLF from human milk (Sigma); fluorescent dye Rhodamine 6G, histidine, and mannitol (Reanal); other reagents were of extrapure grade.

LF was isolated from colostrum (obtained during the first 4 days of lactation; fresh or stored at -20°C).

LF was isolated and purified by ion-exchange chromatography on Servacel CM-52 or gel filtration on Toyopearl HW-52 columns [7].

LF was saturated with iron in a buffer containing 0.1 M sodium citrate and 0.1 M $NaHCO_3$ (pH 8.5, buffer A); 1.2 ml 1.1% LF was titered by adding 20 μl of a solution containing 20 mM Fe^{3+} in buffer A and spectrophotometrically measuring optical density at 470 nm. The content of LF-bound iron was determined by the phenanthroline method (serum iron assay) [1].

Chemiluminescent (CL) analysis of reactive oxygen species was carried out in model H_2O_2—Fe^{2+} systems.

To this end, 200 μl 50 mM Na-phosphate buffer (pH 7.4, buffer B), 100 μl H_2O_2 (0.1 or 0.01%) and 50 μl $FeSO_4 \times 7H_2O$ (final concentrations 5×10^{-2} and

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5×10^{-3} M) were mixed in a thermostatic cuvette (37°C) for 5 min.

In experimental cuvettes, iron-free LF or LF saturated with iron by approximately 45% (10.7 μl Fe/g protein) were added (50 μl each) in a buffer containing 50 mM NaH_2PO_4 and 7 mg/ml protein.

CL analysis was carried out on a PKhL-1 thermostatic setup (Moscow); a FEU-39A photomultiplier tube served as a detector.

For investigation of natural free radicals in a H_2O_2—Fe^{2+} systems, 2.5 mg/ml histidine and 4.5 mg/ml mannitol were used as scavengers of singlet oxygen and hydroxyl radicals, respectively. H_2O_2 was added to a final concentration of 0.1%, the concentration of $FeSO_4$ did not exceed 5×10^{-3} M.

CL analysis was carried out on a setup for recording of induced CL (Institute of Biology, Rostov State University) constructed on the basis of a scintillation spectrometer 22028 (RFG); a FEU-37 photomultiplier tube served as a detector.

Suspension of yolk lipoproteins was prepared by diluting egg yolk in distilled water (1:10). Fluorescent dye rhodamine 6G was added for activation of Fe^{2+}-induced CL [6].

Measuring cell contained 200 μl yolk suspension, 50 μl 1 mM rhodamine 6G, 3 ml 25 mM K-phosphate buffer (pH 7.7), and 100 μl LF solution (5 mg/ml). The baseline CL was recorded for 100 sec, after that 500 μ 25 mM $FeSO_4$ was added to the system and the dynamics of Fe^{2+}-induced CL was recorded.

The antioxidant effect of LF was assessed by the total yield of fast and slow flashes during 100 sec after introduction of $FeSO_4$ and by the amplitude of slow flash and the latency of CL.

RESULTS

Increasing the concentration of Fe^{2+} by one order of magnitude enhanced CL 3- and 2.5-fold at H_2O_2 concentrations of 0.1 and 0.01%, respectively (Table 1). The addition of H_2O_2 induced Fenton reaction nearby iron ions: $Fe^{2+}+H_2O_2\rightarrow Fe^{3+}+OH^{\bullet}+OH^{-}$. Iron ions catalyze this reaction, and its rate depends on the concentration of Fe^{2+}. The presence of LF in the H_2O_2—Fe^{2+} system abolished CL flash. The addition of apoLF to the system containing 5×10^{-3} M $FeSO_4$ and 0.1% H_2O_2 reduced the intensity of CL 6.4-fold, and in the system containing 0.01% H_2O_2 — 45-fold (Table 1). In systems containing 0.1 and 0.01% H_2O_2, LF reduced the intensity of CL 4.7- and 3.18-fold, respectively.

Higher antioxidant activity of apoLF in comparison with LF was due to its higher Fe^{3+}-binding capacity. Since the reaction $Fe^{3+}+O^{2+}\rightarrow Fe^{2+}+O_2$ was reversible, binding of Fe^{3+} prevented initiation of free radical processes. LF eliminated Fe^{3+} from the reaction medium and prevented regeneration of Fe^{2+}. This was confirmed by higher efficiency of apoLF in comparison with LF partially saturated with Fe^{3+} and possessing a lower iron-binding capacity.

The presence of LF in the medium containing 5×10^{-2} M $FeSO_4$ less effectively reduced the intensity of CL (Table 1). In the medium containing 0.1 and 0.01% H_2O_2, apoLF reduced the amplitude of fast flash 2.2- and 5.9-fold, respectively, while LF decreased this parameter 1.9- and 3.9-fold, respectively. The iron-binding capacity of the studied proteins was completely realized at lower concentrations of $FeSO_4$.

Histidine and mannitol reduced the intensity of CL 3-fold (Table 2). The presence of apoLF decreased the amplitude of fast flash 7.5-fold. However, addition of histidine and mannitol in combination with apoLF did not affect the reaction.

These findings suggest that apoLF is a more potent antiradical agent than well-known scavengers of free radicals, and apart from binding of Fe^{2+} and Fe^{3+}, it decreased the content of $OH^{\bullet}$ in model systems.

The addition of LF (45% saturation with iron) to a model system containing yolk lipoproteins, Fe^{2+}, and rhodamine 6G reduced the intensity of fast flash by

TABLE 1. Intensity of Fast Flash of CL in H_2O_2—Fe^{2+} System ($M\pm m$, $n=5$)

Medium	Concentration of H_2O_2, %	
	0.1	0.01
Buffer B+5×10^{-3} $FeSO_4$	84.7±1.45	106.0±2.4
Buffer B+5×10^{-2} $FeSO_4$	260.0±5.16*	271.0±12.4*
Buffer B+apoLF	3.67±0.33	3.33±0.33
Buffer B+LF	3.33±0.33	2.33±0.33
Buffer B+5×10^{-3} $FeSO_4$+apoLF	13.3±1.2*	2.33±0.33
Buffer B+5×10^{-3} $FeSO_4$+LF	18.0±0.57*	33.3±0.33*
Buffer B+5×10^{-2} $FeSO_4$+apoLF	119.0±8.21*	46.7±2.33
Buffer B+5×10^{-2} $FeSO_4$+LF	139.0±2.08*	18.0±0.57*

Note. Here and Table 2: *$p<0.01$, **$p<0.001$ compared with the control.

TABLE 2. Effect of Scavengers of Singlet Oxygen and Hydroxyl Radicals on Intensity of CL Fast Flash (I_{max}) in H_2O_2—Fe^{2+} System ($M\pm m$, $n=5$)

Medium	Agents	I_{max}, rel. units
Buffer B+0.1% H_2O_2+	—	97.0±3.92
5×10^{-3} $FeSO_4$	Mannitol	31.6±2.47*
	Histidine	34.3±1.83*
Buffer B+0.1% H_2O_2+	—	13.0±1.09**
5×10^{-3} $FeSO_4$+apoLF	Mannitol	11.7±2.13*
	Histidine	12.0±1.11*

TABLE 3. Effect of LF Saturation with Iron on Free Radical Oxidation of Yolk Lipoproteins in Fe^{2+}-Rhodamine 6G System ($M \pm m$)

Saturation, %	Total yield of fast flash, $\times 10^3$, arb. units	Latency, sec	Total yield of slow flash, $\times 10^3$, arb. units	Amplitude of slow flash, mm
Control (without LF)	448.8±9.051	98.3±7.05	3386.4±50.170	115.5±8.62
45	280.3±6.230	128.2±9.91	1910.0±39.450	72.6±6.18
100	342.4±7.55	108.1±9.34	2328.2±44.9	85.5±5.90

37.14% (Table 3) The antioxidant properties of LF completely saturated with iron were reduced by 15.38% in comparison with partially saturated LF. The intensity of CL in the presence of completely saturated LF was decreased by 25.7% in comparison with the control.

Thus, LF from breast milk produced a pronounced antiradical effect, which is realized via different mechanisms.

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