Comparative Study of Antioxidant Properties of Immunoregulatory Peptides

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 136, No. 8, pp. 179-183, August, 2003
Original article submitted May 14, 2003

We studied antioxidant properties of immunofan, bursin, cyclobursin, thymopoietin II fragment, glycine, and Siberian ginseng. Experiments were performed in 2 model systems: Fe^{2+}-induced oxidation of multilamellar phospholipid liposomes in a heterogeneous water-lipid system and oxidation of luminol induced by α,α'-azo-bis(isobutyramidine dihydrochloride) in a homogenous aqueous system. By the ability to entrap lipid peroxyl radicals, antioxidant activity of substances decreased in the following order: Siberian ginseng extract>bursin>cyclobursin>thymopoietin II fragment>immunofan, glycine. Siberian ginseng extract and thymopoietin II fragment interacted with Fe^{2+}, which contributed to elimination of catalyst of lipid peroxidation from the system. The ability of substances to interact with aqueous peroxyl radicals and luminol radicals decreased in the following order: Siberian ginseng extract>thymopoietin II fragment>immunofan>glycine, cyclobursin, bursin. Substances with high antioxidant activity improved the state of the endogenous antioxidant system and protected cells from oxidative stress. They entrapped reactive oxygen species formed in the cytoplasm, modulated free radical processes, and regulated the synthesis of bioactive molecules.

Key Words: *reactive oxygen species; antioxidant activity; chemiluminescence; immunoregulatory preparations*

The concept of free radical mechanisms underlying induction of the synthesis of cytokines, growth factors, and proteins (superoxide dismutase, catalase, and inducible NO synthase) and cell proliferation was formulated and experimentally confirmed in the last decade [6]. this concept is based on the assumption that chemical (lipopolysaccharide, cytokines, and growth factors) and physical (ionizing and UV radiation, laser irradiation, and magnetic fields) inductors of protein synthesis first cause the formation of reactive oxygen species (ROS, *e.g.*, H_2O_2) in the cytoplasm [5]. The formation of these radicals is followed by activation of cytosolic transcription factors. Activated NF-kB is translocated from cell cytoplasm into the nucleus, binds to DNA, initiates some gene regions, and stimulates the synthesis of proteins and cytokines.

Experiments with free radical traps (*e.g.*, glutathione, dithiocarbamate, α-tocopherol, N-acetyl-L-cysteine, ascorbate, tairon, and flavonoids) demonstrated the important role of ROS in the initiation of synthetic processes [7,8]. Free-radical pathway of induction of the synthesis of regulatory proteins and cytokines and cell proliferation is involved in the pathogenesis of various diseases, including immune disorders, tumors, and atherosclerosis. It should be emphasized that ROS in low concentrations (μM) initiate intracellular synthetic processes. Therefore, antioxidants in low concentrations are required to scavenge these agents.

Pathogenetic preparations (*e.g.*, regulatory peptides) initiating the synthesis of proteins and cytokines and characterized by a dual mechanism of action are of particular therapeutic importance. On the one hand, they trigger the synthesis and/or proliferation and, thus

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0007-4888/03/1362-0155$25.00

stimulating intracellular ROS generation. On the other hand, these preparations entrap radicals and inhibit synthetic processes.

Antioxidant activity of individual compounds or their mixtures is studied with several model oxidation systems differing by the phase state and mechanisms of generation of certain radicals: homogenous aqueous, hydrophobic homogeneous and hydrophobic heterogeneous systems (suspension of membranes or lipoproteins) [1].

Here we evaluated activity and antioxidant properties of various immunoregulatory compounds.

MATERIALS AND METHODS

We used α,α'-azo-bis(isobutyramidine dihydrochloride) (AIBA, Fluka), luminol (Serva), 2,3,5,6-1H,4H-tetrahydro-9-(2-benzimidazolyl)quinolysino-(9,9a,1-gh)-coumarin (C-525, NIOPIK), FeSO4×7H2O (chemically pure grade), tris(hydroxymethyl)-aminomethane (pure grade, Reakhim), KCl (chemically pure grade), methanol (chemically pure grade), chloroform (chemically pure grade), acetone (extra pure grade), and hexane (pure for analysis grade, Khimmed). Solvents were distilled before the experiment.

The regulatory peptide Immunofan (Bionoks) and new immunoregulatory peptides bursin (synthetic hexapeptide, lysil-histidyl-glycyl-lysil-histidyl-glycine), cyclobursin (synthetic cyclo-hexapeptide, cyclo-lysil-histidyl-glycyl-lysil-histidyl-glycine), and thymopoietin II fragment with amino acid residues in positions 29-41 served as substances with possible antioxidant activity. Glycine (Merck) and atomized extract of Siberian ginseng (Relikt) were used as non-peptide compounds affecting reticuloendothelial and immune cells.

Antioxidant properties were studied in 2 model systems: homogenous aqueous system of luminol oxidation induced by azocompound AIBA (AIBA-luminol system) and heterogeneous system of oxidation of the suspension from phospholipid liposomes induced by Fe^{2+} (Fe^{2+}-liposome system) [1].

The suspension of multilamellar liposomes was prepared from total fraction of yolk phospholipids. Total phospholipids were extracted by the method of J. Folch *et al*. [4]. Multilamellar phospholipid liposomes were prepared by the method of A. D. Bangham *et al*. [3]. The suspension of liposomes was subjected to cryolytic treatment (3-fold freezing and defrosting) to obtain liposomes of the same size. The suspension of liposomes was kept at 37°C for at least 1 h.

Chemiluminescence (CL) was measured on a KhLM-3 chemiluminometer (Bikap). Fe^{2+}-induced CL of the liposome suspension was estimated in the reaction medium (5 ml) containing 0.05 ml liposome suspension (final phospholipid concentration 0.4 mg/ml) and 20 mM Tris-HCl buffer (pH 7.4). CL was initiated by C-525 (coumarin derivative) dissolved in ethanol (final concentration 0.5 μM). Lipid peroxidation (LPO) was induced with $FeSO_4$ (final concentration 7 μM). Kinetic parameters of CL were recorded on a Macintosh LCII computer equipped with a MacLab/2e (ADInstruments) interface for 10-30 min.

Antioxidant properties of regulatory compounds were also studied in the homogenous aqueous system with AIBA. Generation of peroxyl radicals $RO_2^{\bullet}$ accompanying thermal decomposition of AIBA in the

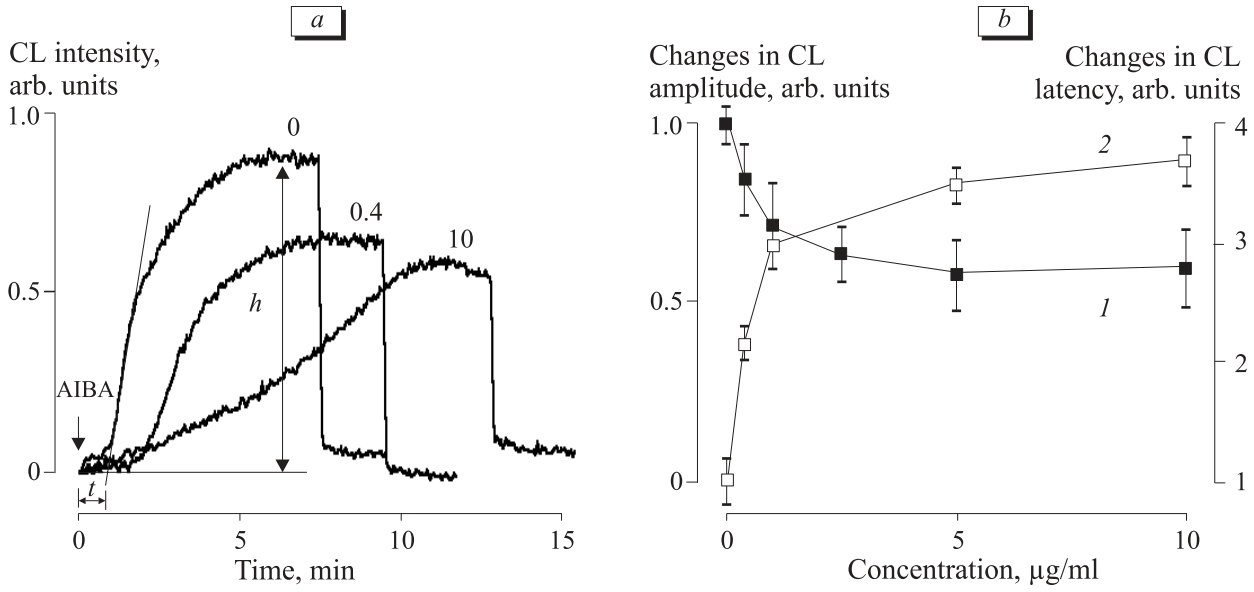

Fig. 1. Effect of Siberian ginseng extract (SGE) on chemiluminescence (CL) in the AIBA-luminol system. *a*) kinetics of CL without and with SGE. Figures near curves: SGE concentration (μg/ml). *t*, latency of CL; *h*, maximum intensity of luminol-dependent CL. Arrow: addition of AIBA. *b*) effect of SGE on the amplitude (*1*) and latency of CL (*2*).

model system was determined by luminol-dependent CL (LCL) [1]. The incubation medium (5 ml) contained 5 mM AIBA and 10 μM luminol in phosphate buffer. AIBA (5 mM) was added to the incubation medium containing phosphate buffer (50 mM KH_2PO_4 and 100 μM, pH 7.4) and 10 μM luminol at 37°C under constant mixing. The medium volume was 5 ml. Control CL was measured. In experimental samples test substances (in phosphate buffer) were introduced into the incubation medium before AIBA addition.

RESULTS

The homogenous aqueous system with AIBA is used to measure antioxidant activity, since this azocompound undergoes thermal degradation into 2 peroxyl radicals $RO_2^{\bullet}$. The interaction of peroxyl radicals with luminol produces LCL (Fig. 1, *a*). The radical-entrapping agent added to the system competes with luminol for radicals. This process is accompanied by quenching of CL and the appearance of a lag-period (time required for complete utilization of the antioxidant).

Activity of antioxidants also depends on the maximum intensity of LCL. The decrease in the intensity of LCL in the model system can be related to the interaction of antioxidants with luminol radicals.

We evaluated the effect of Siberian ginseng extract (SGE) on CL in the model system of AIBA and luminol (Fig. 1, *a*). Increasing the concentration of SGE was accompanied by lengthening of its latency in chemiluminogram and decrease in its amplitude. Titration of the model system confirmed our hypothesis. The preparation in a concentration of 5 μg/ml caused 50% quenching of LCL in the model system (Fig. 1, *b*, curve *1*). Measurements of chemiluminescence latency revealed a biphasic effect of SGE (Fig. 1, *a*, curve *2*). At the beginning of titration SGE in concentrations below 1 μg/ml increased chemiluminescence latency by more than 2-fold. These changes reflect the ability of this compound to entrap peroxyl radicals in the model system. The latency reached a plateau after treatment with SGE in concentrations of 1-5 μg/ml. Further increase in SGE concentration to 10 μg/ml was accompanied by its insignificant lengthening.

We evaluated the concentrations at which the test substances produced most significant changes in LCL of the AIBA-luminol system (Table 1). The ability of substances to interact with radicals in the model system decreased in the following order: SGE>thymopoietin II fragment>immunofan>glycine, cyclobursin, bursin.

Antioxidant activity of preparations was also estimated in the heterogeneous system of multilamellar liposomes. Free radical LPO in liposomal membranes was induced with Fe^{2+}. Coumarin C-525 that acts as a physical activator of luminescence and laser dye was introduced into the system to increase the intensity of CL during LPO [2].

We analyzed 2 main parameters of chemilimunogram: latency and amplitude of slow flash.

TABLE 1. Effect of Test Substances on CL in Model Oxidation Systems

Substance, concentration		Fe^{2+}-liposomes		AIBA-luminol	
		H	τ	h	t
Control	0	1.00	1.00	1.00	1.0
CycloBursin	220 μM	0.75	1.20		
	600 μM	0.69	1.36	1.00	1.0
Bursin	3 μM	0.75	1.00		
	600 μM	0.48	1.13	1.00	1.0
Glycine	230 μM	0.90	1.00	1.00	1.0
Immunofan	3 μM			0.75	1.0
	470 μM	0.82	1.00		
Thymopoietin fragment	23 μM			0.75	1.4
	30 μM	0.75	0.93		
	62 μM	0.79	0.83		
SGE	0.7 μg/ml			0.75	2.7
	2.5 μg/ml	0.75	1.00		
	40 μg/ml	0.07	0.39		

Note. H and τ are the slow flash amplitude and latency of CL in the Fe^{2+}-liposome system, respectively; *h* and *t* are the amplitude and latency of CL in the AIBA-luminol system, respectively. Parameters of CL are expressed in relation to the control value (without test substances).

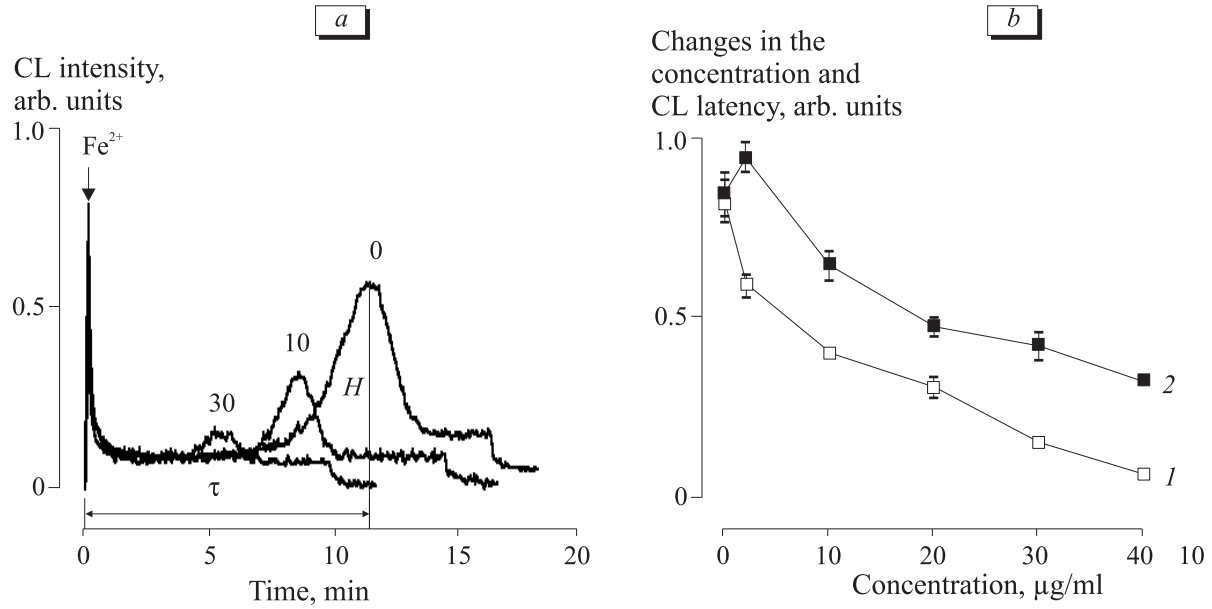

Fig. 2. Effect of SGE on CL in the Fe^{2+}-liposome system. *a*) kinetics of CL without and with SGE. Numerals near curves: SGE concentration (μg/ml). τ, latency of CL; *H*, slow flash amplitude. Arrow: addition of Fe^{2+}. *b*) effect of SGE on the amplitude (*1*) and latency of CL (*2*).

Chemiluminiscence latency depends on the influence of substances on Fe^{2+} concentration. They include chelators (complex-forming substances) and compounds that affect the state of iron ions (oxidizing and reducing agents). EDTA, phosphates, and desferral chelate and form stable complexes with Fe^{2+}, where iron ions exhibit no catalytic activity. These substances added to the system shorten the latency. Published data show that agents oxidizing Fe^{2+} into Fe^{3+} produce similar changes (*e.g.*, ceruloplasmin). By contrast, various agents (*e.g.*, ascorbate) reducing Fe^{3+} into Fe^{2+} and responsible for regeneration of the catalyst increase the latency of chemiluminogram in the suspension of liposomes.

It should be emphasized that the latency increases in the presence of $RO_2^{\bullet}$-entrapping substances. In other words, the decrease in the intensity of slow flash in the presence of antioxidants can be accompanied by lengthening of CL.

We evaluated the effects of SGE on coumarin-induced CL in the suspension of liposomes (Fig. 2, *a*).

Increasing the concentration of SGE in the model system was accompanied by a decrease in the amplitude of slow flash in chemiluminogram and shortening of its latency. Titration of the liposomal suspension confirmed these observations (Fig. 2, *b*). SGE in concentrations of 10-30 μg/ml changed parameters of CL by 50%. The addition of SGE during quenching of luminescence in the model system decreased chemiluminescence latency. Our results indicate that this compound not only entraps aqueous and, probably, hydrophobic radicals, but also interacts (chelates and/or oxidizes) with Fe^{2+} catalyzing free radical reactions.

Antioxidant activity of substances decreased in the following order: SGE>bursin>cyclobursin>thymopoietin II fragment>immunofan, glycine (Table 1).

Regulatory compounds with antioxidant activity play an important role in the pathogenesis of various inflammatory diseases. First, substances with high antioxidant activity and other compounds of the endogenous antioxidant system determine antioxidant status of the organism (*e.g.*, in the blood) [1]. And second, immunoregulatory compounds and peptides capable of entrapping free radicals in the cell cytoplasm determine the free radical mechanism of synthetic processes (superoxide dismutase, catalase, inducible NO synthase, cytokines, and growth factors).

REFERENCES

1. G. I. Klebanov, *Ros. Zh. Gastroenterol. Gepatol. Koloproktol.*, **11**, No. 4, 109-118 (2002).
2. V. S. Sharov, E. S. Dremina, and Yu. A. Vladimirov, *Biofizika*, **40**, No. 2, 248-433 (1995).
3. A. D. Bangham, J. De-Gier, and D. Greville, *Chem. Phys. Lipids*, **1**, No. 2, 225-246 (1967).
4. J. Folch, S. M. Lee, and G. Y. Sloane-Stanley, *J. Biol. Chem.*, **226**, No. 1, 497-509 (1957).
5. I. A. Gamaley, K. M. Kirpichnikova, and I. V. Klyubin, *Cell Signal*, **6**, No. 8, 949-957 (1994).
6. M. Los, W. Droge, K. Stricker, *et al.*, *Eur. J. Immunol.*, **25**, No. 1, 159-165 (1995).
7. X. Shi, Z. Dong, Ch. Huang, W. Ma, *et al.*, *Mol. Cell Biochem.*, **194**, No. 1, 63-70 (1999).
8. H. W. Zeigler-Heitbrock, T. Sternsdorf, J. Leise, *et al.*, *J. Immunol.*, **151**, No. 12, 6986-6993 (1993).