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PHYSIOLOGY

Comparison of Antioxidant Properties of Melatonin, Epithalamin, and Glutathione by *in vitro* Luminol-dependent Chemiluminescence

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Oxidative stress, which is characterized by the generation of excessive amounts of reactive oxygen species (ROS), may be induced in an organism under various conditions. Thus, development of ischemia is accompanied by activation of the xanthine–xanthine oxidase system and inhibition of superoxide dismutase (SOD) and glutathione peroxidase [1]. ROS exhibit a cytotoxic effect; they alter the fluidity and permeability of membranes and induce peroxidation of membrane phospholipids and proteins, thus damaging the membranes [2]. Tissue damage resulting from free-radical oxidation is now believed to be one of the causes of aging [3, 4]. In view of this, the antioxidant properties of melatonin are of particular interest. In mammals, melatonin is secreted by the epiphysis; it suppresses the development of tumors, activates the immune system, and increases life span [5–7]. It was found that inhibition of *in vitro* generation of the highly toxic hydroxyl radical by melatonin is 5–14 times higher than inhibition by glutathione or mannitol [8]. We found that melatonin efficiently inhibited free-radical peroxidation in rats both *in vivo* and *in vitro* [7, 9, 10].

This work was designed to compare the antioxidant properties of melatonin and epithalamin, a peptide preparation from the bovine epiphysis [10], based on quenching of the luminol-dependent chemiluminescence (CL) excited either by an ROS compound (hypochlorite OCl^-, superoxide O_2^-, or hydrogen peroxide H_2O_2) or by ROS generated *in vitro* by activated neutrophils.

Measurements were performed on an LKB luminometer (Sweden) at 25°C in a reaction mixture (1 ml; pH 7.4) containing 0.01 M Na_2HPO_4, 0.01 M Na_2HPO_4, 0.15 M NaCl, and 2×10^{-5} M luminol (Fluka). The final concentrations of hypochlorite or hydrogen peroxide in the reaction mixture were 10^{-7} and 5×10^{-5} M, respectively. Superoxide anion was obtained by conversion of 0.25 mM xanthine with xanthine oxidase (125 U/ml).

Polymorphonuclear leukocytes were isolated from human blood by centrifugation in a Ficoll density gradient [12]. Neutrophils were activated by addition of 1 μg of phorbol myristate to 1 ml of the neutrophil suspension (10^6 cells/ml) in a medium containing 0.14 M NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.1% glucose, and 0.1% gelatin (pH 7.4) at 37°C.

When CL was induced by hypochlorite, the maximum CL intensity was observed during the first second of the reaction. The results were calculated considering the total light emitted over 2 s. The CL intensity upon the ROS (hypochlorite) interaction with luminol was taken as 100% (control). When hydrogen peroxide was added to the reaction mixture, the maximum CL intensity was observed at the third second. The results were calculated considering the emitted light measured over 6 s.

The addition of 10^{-5} M melatonin, 25 μg/ml epithalamin, or 10^{-5} M glutathione to the reaction mixture decreased the CL intensity, probably due to the competition of these compounds with luminol for free radicals.

The maximum quenching (up to 30%) of the hypochlorite-induced CL was observed when melatonin was added to the reaction mixture. Epithalamin was less efficient (up to 17%), and glutathione, a widely known antioxidant, exhibited the lowest capacity for competition for hypochlorite (up to 12% quenching of CL).

Figure I shows that in the presence of hydrogen peroxide, melatonin quenched CL more efficiently than did glutathione (45 and 20%, respectively). The epithalamin activity in the presence of hydrogen peroxide (33%) was higher than in the presence of hypochlorite.

When the superoxide radical was generated in the xanthine oxidase reaction, the maximum CL intensity in the control was observed in 2 s. In the presence of melatonin, the maximum CL intensity was observed in 3 s, probably

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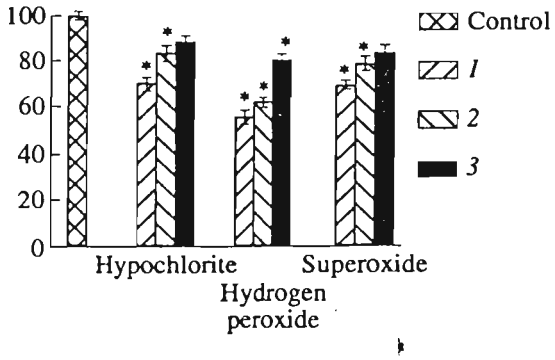

Fig. 1. Quenching of the luminol-dependent chemiluminescence induced *in vitro* by hypochlorite, hydrogen peroxide, or superoxide in the presence of (*1*) melatonin, (*2*) epithalamin, and (*3*) glutathione; *y*-axis, total CL, arbitrary units; asterisk, $P < 0.05$. Each experiment was repeated four times.

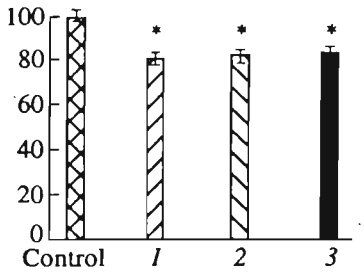

Fig. 2. Quenching of the luminol-dependent chemiluminescence induced *in vitro* by activated neutrophils in the presence of (*1*) melatonin, (*2*) epithalamin, and (*3*) glutathione; *y*-axis, total CL, arbitrary units; asterisk, $P < 0.05$. Each experiment was repeated three times.

due to the action of melatonin on the components of the xanthine oxidase reaction.

Based on the results obtained, the tested compounds may be arranged as follows with respect to their antioxidant activity: melatonin (up to 30%), epithalamin (up to 22%), and glutathione (up to 16.5%).

When CL was induced by activated neutrophils, the addition of melatonin, epithalamin, or glutathione decreased the CL intensity by as much as 20% (Fig. 2), which correlates well with the results reported in [13]. However, the effects of these compounds were considerably less pronounced than when CL was induced by certain ROS.

Our experiments showed that the acceptor activity of melatonin is higher than those of epithalamin and glutathione. This difference may be explained by the structural properties of the studied compounds. The antioxidant properties of melatonin appear to be related to the presence of indole heterocycle in its structure. When interacting with ROS *in vitro*, melatonin donates an electron to these electrophilic compounds, and the generated indolyl cation is converted into a kynuramine derivative in the presence of the superoxide radical. Earlier, we showed that under similar conditions, the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) also exhibit antioxidant properties. Each of these hormones contains two phenol groups that display both electron-acceptor and electron-donor properties [14], and the antioxidant activities of these compounds in the model system used were even higher than that of melatonin. Therefore, nonspecific hormones, such as T_3, T_4, and melatonin, may inhibit free-radical processes in an organism. The *in vitro* antioxidant activity of epithalamin may be related to the presence of peptide groups and aromatic amino acids in this compound. It is known that serum proteins, in particular, ceruloplasmin, serve as traps for oxygen intermediates [15]. Glutathione in its oxidized and reduced forms exhibits either electron-donor or electron-acceptor properties.

In conclusion, we obtained new evidence on the antioxidant properties of melatonin and epithalamin. Epithalamin was a less efficient antioxidant than melatonin *in vitro*; however, *in vivo*, the effect of epithalamin was more pronounced than that of melatonin, possibly due to the epithalamin-stimulated biosynthesis and secretion of melatonin in animals [11].

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