Inhibition of Low Density Lipoprotein Oxidation by Melatonin

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The antioxidant activity of the lipophilic hormone melatonin, "an ideal inhibitor of free radicals," is studied in models of cellular (peritoneal mouse macrophages) and copper-induced oxidation of low density lipoproteins. Oxidative modification of low density lipoproteins is assessed by accumulation of thiobarbituric acid reactive substances and degradation of ^{125}I-labeled lipoproteins in a fresh culture of macrophages. Melatonin inhibits in a dose-dependent manner cellular and copper-induced oxidation of lipoproteins and production of the superoxide anion radical by macrophages, the mean concentrations of 50% inhibition being 300, 1230, and 900 μM, respectively.

Key Words: *oxidation of low density lipoproteins; melatonin; macrophage*

The uptake of oxidized low density lipoproteins (LDL) by monocytes and macrophages with subsequent formation of foam cells is a key event in atherogenesis [14]. Oxidation of LDL was observed in the presence of transition metals (Cu^{2+} and Fe^{2+}), during irradiation with ultraviolet light, and upon incubation with phagocytes, endotheliocytes, and smooth muscle cells [5]. Numerous *in vitro* and *in vivo* studies proved that LDL are oxidized by the free radical pathway; however, the participation of some enzyme systems and activated oxygen metabolites (O_2^-, $OH^\cdot$, HOCl, $RO_2^\cdot$, $NO^\cdot$, etc.) in this processes is debated [3,5,14].

In mammals, the indoleamine melatonin (N-acetyl-5-methoxytryptamine) is produced by the pituitary gland in darkness. The unique antioxidant properties of melatonin make it an "ideal inhibitor of free radicals" [9]. It was demonstrated that this hormone effectively inhibits OH radical [12] and NO synthetase [8]. In animal experiments, melatonin inhibited the development of alloxan-induced diabetes, displayed anti-inflammatory activity, reduced DNA damage, prevented malignization of cells, and markedly prolonged life span [6,7,9]. All these pathological processes are associated with free radical oxidation and damage to biological structures.

Based on these findings, we hypothesized that melatonin modulates oxidation of LDL in mammals. To test this hypothesis we examined the protective effect of melatonin on cellular and Cu^{2+}-induced oxidation of LDL and assessed its ability to inhibit the production of O_2^- by macrophages.

MATERIALS AND METHODS

Low density lipoproteins (d=1.019-1.055 g/ml) were isolated from the plasma of normolipidemic donors by ultracentrifugation and labeled with ^{125}I as described elsewhere [1].

Resident peritoneal macrophages were obtained from C57Bl/6J mice weighing 16-18 g. The cells were washed and incubated in 40-mm plastic dishes for 24 h. Macrophage monolayers were used to study oxidative modification of LDL, degradation of ^{125}I-LDL, and chemiluminescence [1]. For cellular oxidation the macrophage monolayer was incubated

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for 24 h at 37°C in Ham's F-10 medium containing 50 μg/ml gentamicin, 0.01% lipoprotein-deficient serum, and 200 μg/ml LDL or 50 μg/ml ^{125}I-LDL. Copper-induced oxidation of LDL (280 mg/ml) was performed in Ca^{2+}- and Mg^{2+}-free Dulbecco's medium by a 6-h incubation with 10 μM $CuSO_4$ at 37°C. Melatonin (Sigma) was dissolved in ethanol and added immediately before incubation. Oxidative modification of LDL was assessed by measuring the concentration of thiobarbituric acid reactive substances (TBARS) [10] and the rate of ^{125}I-LDL degradation by macrophage monolayer cultured for 5 h at 37°C [11].

Chemiluminescence was measured as described elsewhere [1]. Cell monolayers were placed in a thermostatically controlled (37°C) cell of a chemiluminometer after washing with Hanks' medium free from phenol red and addition of 2 ml 10^{-4} M lucigenin. Spontaneous luminescence was recorded for 3 min, after which chemiluminescence was measured for 3 min in the presence of 10 μl ethanol solution of melatonin. Macrophages were then stimulated with 0.2 ml of opsonized zymosan (20 mg/ml), and the intensity of chemiluminescence was measured before it reached the maximum.

The results were processed using a Statgraphics software; the concentration of 50% inhibition (IC_{50}) was determined from the equations for approximating lines obtained by regression analysis.

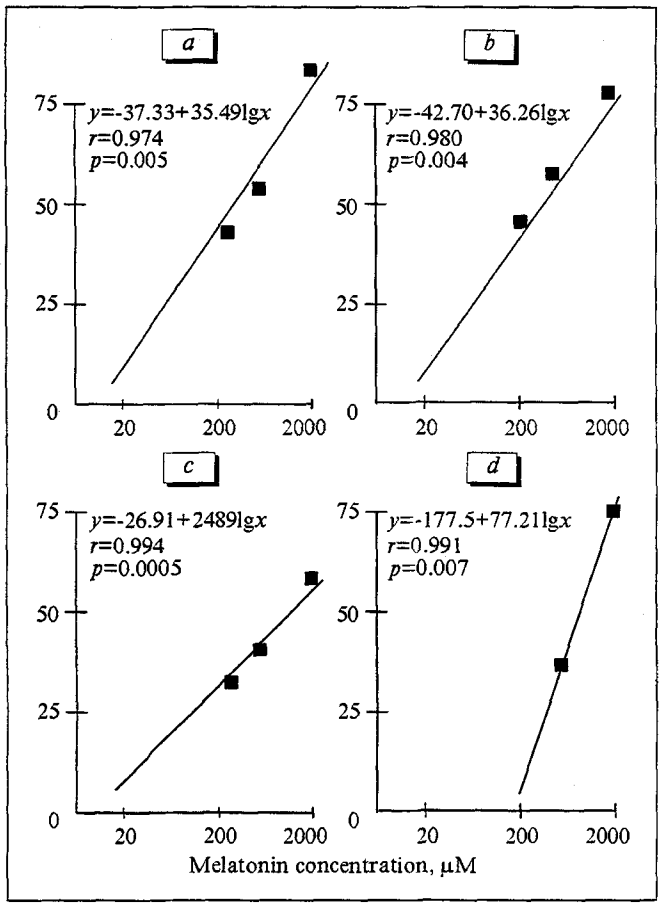

Fig. 1. Inhibition of macrophage- (*a*, *b*) and Cu^{2+}-induced (*c*) oxidation of LDL and the intensity of zymosan-induced chemiluminescence of macrophages (*d*) as a function of melatonin concentration. Ordinate: percent of inhibition of TBARS accumulation in LDL (*a*, *c*), rate of ^{125}I-LDL degradation by macrophages (*b*), and intensities of chemiluminescence (*d*).

RESULTS

Incubation of LDL resident peritoneal macrophages resulted in oxidative modification of LDL, judging from the increased content of TBARS and the rate of ^{125}I-LDL degradation by fresh layer of macrophages (Table 1). Melatonin inhibited cellular oxidation of LDL in a dose-dependent manner: IC_{50} for the accumulation of TBARS and degradation of ^{125}I-LDL by macrophages were 289 and 360 μM, respectively (Fig. 1, *a*, *b*). The inhibitory activity of melatonin toward Cu^{2+}-induced oxidation of LDL

TABLE 1. Effect of Melatonin on LDL Modification by Resident Macrophages

LDL	TBARS content, nmol/mg protein LDL	Degradation, μg protein of ^{125}I-LDL/mg cell protein for 5 h
Native	13.1±0.7	0.28±0.04
Incubated without macrophages	24.3±3.9	1.21±0.06
Incubated with macrophages	65.0±11.8	5.37±0.30
Incubated with macrophages+melatonin:		
20 μM	56.8±3.1	4.85±0.23
80 μM	52.4±5.0	4.33±0.31
250 μM	42.5±3.9*	2.89±0.18*
500 μM	36.4±2.6*	2.28±0.12*
2 μM	20.1±2.2*	1.18±0.06*

Note. *$p<0.01$ in comparison with LDL incubated with macrophages.

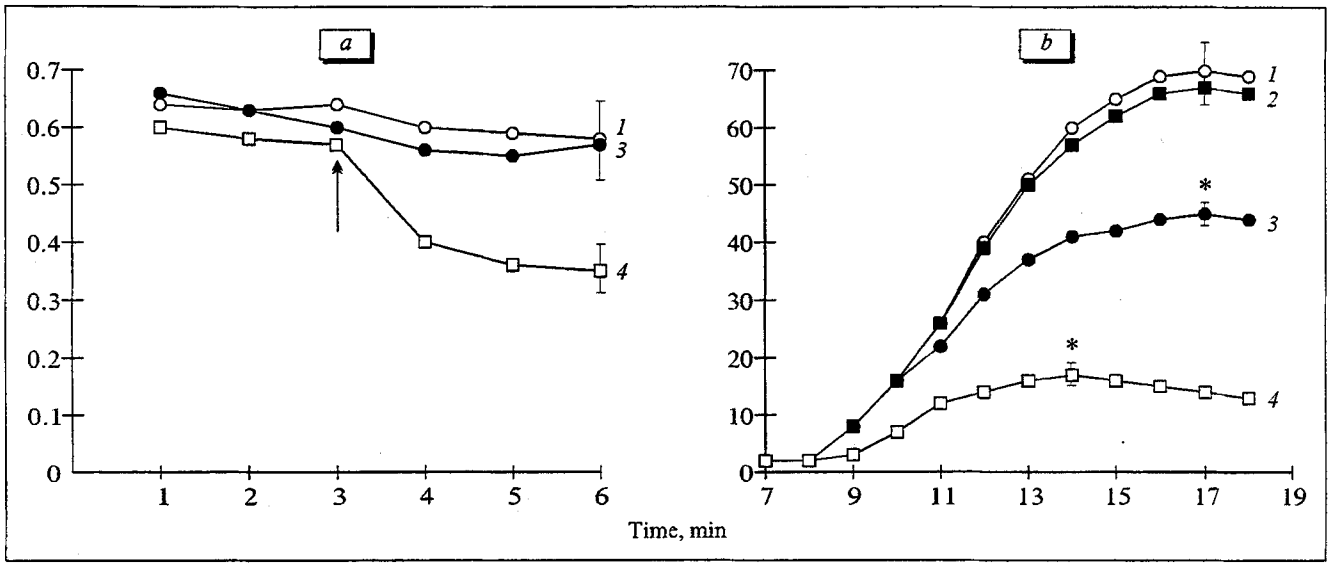

Fig. 2. Effect of melatonin on spontaneous (*a*) and zymosan-induced (*b*) chemiluminescence of macrophages. Ordinate: chemiluminescence intensity (pulses×10^3). Melatonin concentrations: *1*) 0; *2*) 250 μM, *3*) 500 μM *4*) 2 mM. Arrow indicates the addition of melatonin.

was lower: IC_{50}=1230 μM (Fig. 1, *c*). The higher inhibitory activity toward cellular oxidation of LDL may be associated with the production of activated oxygen metabolites (AOM) by macrophages, specifically, O_2^-. In concentrations higher that 200 mM melatonin inhibits the lucigenin-dependent chemiluminescence of macrophages (both spontaneous and zymosan-induced) that reflects the production of O_2^- [1]: IC_{50} being 885 and >2000 μM for zymosan-induced and spontaneous chemiluminescence, respectively (Figs. 1, *d* and 2).

From these findings it can be concluded that the inhibitory effect of melatonin on LDL oxidation *in vivo* is insignificant, since the serum physiological level of this hormone (0.1-0.3 nM) [4] is considerably lower than that required for inhibition of this process. Presumably, OH radical is not directly involved in oxidative modification of LDL, since the concentrations at which melatonin effectively inhibits LDL oxidation are considerably higher than those at which it inhibits the radical (IC_{50}=21 μM [12]). This suggestion is corroborated by the results of others [3,5]. The low protective activity of melatonin toward Cu^{2+}-induced oxidation of LDL (IC_{50} is close to that for brain homogenate oxidation [7]), which supports the hypothesis that oxidative modification of LDL can be initiated by alkoxyl and peroxyl radicals resulting from degradation of organic peroxides by transition metals. This mechanism is confirmed by higher inhibitory activity toward cellular oxidation of LDL, since in this case LDL oxidation depends on endogenous peroxides [13], the formation of which in the macrophage plasma membrane depends on OH radical [2].

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