Inhibition of LDL oxidation by melatonin requires supraphysiologic concentrations

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Melatonin has been suggested as a potent antioxidant that may protect against development of atherosclerosis and cancer; however, these effects are unproven and controversial. The antioxidant capacity of melatonin was tested in comparison with α-tocopherol, ascorbic acid, and the melatonin precursors tryptophan and serotonin, by measuring inhibition of metal ion-mediated and human macrophage-mediated oxidation of LDL. Melatonin had weak antioxidant activity that was detectable only at concentrations 10 000- to 100 000-fold higher than physiologic concentrations. These results were comparable with published data showing that the radical scavenging activity of melatonin requires markedly supraphysiologic concentrations. In contrast, α-tocopherol was 50- to 100-fold more potent and was efficacious at physiologic concentrations. Ascorbic acid and tryptophan also were active at physiologic concentrations and were significantly more potent than melatonin. In summary, extremely supraphysiologic concentrations of melatonin had only weak antioxidant activity, which was surpassed by α-tocopherol, ascorbic acid, and tryptophan.

Melatonin is a lipophilic indoleamine hormone, derived from tryptophan, that is secreted by the pineal gland primarily during periods of darkness (1, 2). It is believed to play a major role in the regulation of diurnal rhythms in vertebrate animals, including humans (2–4). It also has been suggested as a powerful antioxidant that scavenges superoxide, hydroxyl, and peroxy radicals (5–9); however, these effects have been observed primarily at markedly supraphysiologic concentrations. Some investigators have proposed that the antioxidant properties of melatonin may protect against development of cancer, atherosclerosis, and other consequences of aging (10–13); however, these effects remain unproven and controversial (14, 15).

A large body of experimental evidence supports the hypothesis that oxidation of LDL contributes to the development of atherosclerosis (16–21). Moreover, it is postulated that inhibition of LDL oxidation by antioxidants might protect against the development of atherosclerosis (22–27). In both human and animal studies, resistance of LDL to oxidation in vivo often has been associated with decreased atherosclerosis (19, 25, 26, 28). Moreover, antioxidant administration inhibits oxidation of LDL and typically, but not always, has been associated with decreased progression of atherosclerosis (19, 21, 22, 25, 26). The strongest data from human subjects come from the Cambridge Heart Antioxidant Study, in which the risk of cardiovascular death and nonfatal myocardial infarction was reduced 47% in patients with coronary disease who received 400–800 IU of vitamin E daily (27). Because melatonin has been reported to be a powerful antioxidant with possible antiatherogenic properties, we endeavored to test the relative capacity of melatonin, its structurally related precursors, tryptophan and serotonin, and the antioxidant vitamins, α-tocopherol and ascorbic acid, to inhibit oxidative modification of LDL in vitro.

Materials and Methods

Lipoprotein isolation

LDL was isolated from pooled fresh human plasma by sequential ultracentrifugation in the density range 1.019–1.063 kg/L using standard methods (29, 30). LDL was stored in 1 mmol/L EDTA in phosphate-buffered saline (9 g/L NaCl) under nitrogen at 4 °C in the dark and was used within 2 weeks after isolation. EDTA was removed from LDL samples before oxidation assays by extensive dialysis in degassed phosphate-buffered saline (9 g/L NaCl) or desalting with a Sephadex G-25 (PD-10) column (Sigma Chemical Co.) equilibrated with degassed phosphate-buffered saline (9 g/L NaCl).

Cell culture

Human monocyte-derived macrophages were obtained by Ficoll/Hypaque density gradient centrifugation of blood from healthy donors and grown in primary culture in RPMI-1640 culture medium (Gibco BRL) with 200
mL/L autologous serum at 37 °C in humidified incubators containing 5% CO₂/95% air as previously described (30–32). Cells were plated at a density of 1–2 × 10⁶ cells per 22-mm plastic well and used after 7–10 days.

**CELL-FREE LDL OXIDATION**

LDL was oxidized in vitro by incubating 0.52 mmol/L (200 μg/mL) LDL-cholesterol in phosphate-buffered saline (9 g/L NaCl) in the presence of 5 μmol/L copper sulfate at 20 °C for 8 h in a temperature-controlled, multicuvette Shimadzu spectrophotometer. In other experiments, LDL was oxidized by incubation of 1.82 × 10⁻⁷ mol/L (100 μg/mL) LDL protein in Ham’s F-10 medium at 37 °C (Gibco BRL) for 18–24 h. Oxidative modification of LDL was monitored by determining the formation of conjugated dienes by semicontinuous measurements of the absorbance at 234 nm (33, 34) or sequential measurements of thiobarbituric acid-reactive substances (TBARs) (35, 36). These standard methods for determining the susceptibility of LDL to oxidative modification have been used extensively to evaluate the antioxidant properties of various compounds (33, 34). The lag time for LDL oxidation was measured as the intercept of tangent lines for the initiation and propagation phases of the curve showing the time course for formation of conjugated dienes (33, 34). The propagation rate was measured as the slope of the propagation phase during formation of conjugated dienes (33, 34).

**CELL-MEDIATED LDL OXIDATION**

Macrophage-mediated oxidation of LDL was measured by incubating 1.82 × 10⁻⁷ mol/L (100 μg/mL) LDL protein in triplicate 22-mm wells with 1–2 × 10⁶ cells in Ham’s F-10 medium at 37 °C for 18–24 h. Cell-free control wells were used for all conditions. At the end of incubation, oxidation of LDL was arrested by chilling the medium and adding 200 μmol/L EDTA and 40 μmol/L butylated hydroxytoluene. Aliquots were assayed for TBARS content (35, 36) and normalized for the amount of cell protein determined by a modified method of Hartree (37), using a bicinchoninic acid microtiter plate assay (Pierce Chemical Co.). Cell-mediated oxidation was calculated as the difference between TBARS content in cell-containing and cell-free conditions.

**LDL ELECTROPHORESIS**

Electrophoretic mobility of control and modified LDL was assessed by 0.8% agarose gel electrophoresis at pH 8.6 in barbitol buffer (38). LDL was visualized with Sudan black staining (38). Relative electrophoretic mobility was calculated as the ratio of migration of modified LDL compared with control LDL.

**PREPARATION OF ANTIOXIDANTS**

Melatonin and α-tocopherol were dissolved in ethanol. Tryptophan was solubilized in 0.5 mol/L (0.5 N) hydrochloric acid, and serotonin and ascorbic acid were dissolved in distilled water or ethanol. Antioxidant solutions were prepared fresh, protected from light, and added to the incubation medium in concentrations ranging from 0–50 μmol/L (Sigma). Control samples of LDL with equal volumes of diluent were used as the reference measurement in every experiment. The final concentration of ethanol in the incubation medium was ≤20 mL/L. The final concentration of HCl in experiments with tryptophan was ≤0.25 mmol/L. Neither ethanol nor dilute HCl at these concentrations affected the lag time or propagation rate for LDL oxidation.

**STATISTICAL ANALYSIS**

Statistical analyses were done with Mann–Whitney rank sum and t-testing using Sigmastat statistical software (Jandel Scientific). P values <0.05 were considered statistically significant.

**Results**

In a cell-free system, melatonin at concentrations up to 5 μmol/L had no appreciable effect on LDL oxidation measured as copper-mediated conjugated diene formation (Fig. 1). At a maximal concentration of 50 μmol/L melatonin, there was moderate inhibition of LDL oxidation manifested as a 19% ± 9% increase in the lag time (P = 0.036) and 48% ± 16% decrease in propagation rate (P = 0.016). This concentration of melatonin is 10 000- to 100 000-fold greater than peak physiologic plasma concentrations (~45–900 pmol/L) (39) and 50- to 100-fold higher than maximal serum concentrations achieved after large pharmacologic doses of melatonin (39, 40).

In contrast, α-tocopherol, another lipid-soluble antiox-
oxidant (34, 41), was about 50-fold more potent compared with melatonin (*P < 0.001; Fig. 1). At a concentration of 5 μmol/L, α-tocopherol increased the lag time for conjugated diene formation by 47% ± 17% (*P = 0.036 vs control) without significantly affecting the propagation rate. LDL oxidation was essentially abolished in the presence of 50 μmol/L α-tocopherol (data not shown). These concentrations of α-tocopherol are comparable to physiologic serum concentrations of 12–46 μmol/L. Ascorbic acid, an effective water-soluble antioxidant, also was significantly more potent as an antioxidant compared with melatonin (*P <0.001; Table 1).

Comparable differences between the antioxidant capacity of melatonin and α-tocopherol were observed when LDL oxidation was quantified by measuring TBARSs after incubating LDL in Ham’s F-10 medium [containing 3 mmol/L (0.834 mg/mL) FeSO₄·7 H₂O and 10 μmol/L (0.0025 mg/mL) CuSO₄·5 H₂O] for 18 h at 37 °C. α-Tocopherol at a concentration of 50 μmol/L reduced LDL oxidation by 61% ± 4% (*P < 0.001 vs control) whereas equimolar concentrations of melatonin reduced LDL oxidation by only 22% ± 3% (*P < 0.001 vs control and α-tocopherol; Fig. 2).

Because the indole moiety is presumed to be responsible for radical scavenging activity of melatonin, the relative antioxidant potency of tryptophan and serotonin, indole precursors of melatonin, was tested (Table 1). At concentrations <5 μmol/L, neither tryptophan nor serotonin significantly influenced the lag time for LDL oxidation (data not shown). However, at a concentration of 5 μmol/L, the antioxidant activity of tryptophan was much greater than equimolar amounts of melatonin, producing a 12% ± 9% increase in the lag time compared with −2% ± 9% for melatonin (P = 0.045; Table 1). In contrast, 5 μmol/L serotonin appeared to enhance LDL oxidation, producing a consistent 40% ± 5% decrease in the lag time (P = 0.016). At a concentration of 50 μmol/L, tryptophan increased the lag time by 117% ± 22% (P = 0.016), whereas serotonin completely inhibited LDL oxidation during the 8-h incubation.

To test the effects of melatonin on cell-mediated oxidation, primary cultures of human monocyte-derived macrophages and cell-free control wells were incubated with 1.82 × 10⁻⁷ mol/L (100 μg/mL) LDL protein for 18 h at 37 °C in Ham’s F-10 medium. α-Tocopherol at a concentration of 50 μmol/L reduced oxidation of LDL measured by TBARSs by 87% ± 10% (*P < 0.001 vs control or melatonin), whereas equimolar concentrations of melatonin reduced LDL oxidation by only 21% ± 10% (P = 0.039 vs control; Fig. 3).

To assess the effects of melatonin on apolipoprotein B modification, the relative electrophoretic mobility of LDL was determined after cell-mediated and cell-free oxidation of 1.82 × 10⁻⁷ mol/L (100 μg/mL) LDL protein in Ham’s F-10 medium for 18 h at 37 °C (Fig. 4). Mobility of LDL incubated with human monocyte-derived macrophages was unaffected by 50 μmol/L melatonin, whereas equimolar α-tocopherol prevented 38% of increased mobility of modified LDL. Under cell-free conditions, 50 μmol/L melatonin blocked 16% of increased mobility of modified LDL; however, α-tocopherol blocked 56% of the increase.

**Table 1. Effect of melatonin, melatonin precursors, and other antioxidants on the lag time and propagation rate for conjugated diene formation during copper ion-mediated LDL oxidation.**

<table>
<thead>
<tr>
<th>Change in lag time, % of control</th>
<th>Change in propagation rate</th>
<th>Physiologic serum concentration</th>
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<tbody>
<tr>
<td></td>
<td>5 μmol/L</td>
<td>50 μmol/L</td>
</tr>
<tr>
<td>Melatonin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>12 ± 9*</td>
<td>117 ± 22*</td>
</tr>
<tr>
<td>Serotonin</td>
<td>−40 ± 5*</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>37 ± 7*</td>
<td>94 ± 14*</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>47 ± 17*</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

* Values indicate percentage of change in lag time or propagation rate ± SD and represent data from three to six separate experiments.

*P <0.05 compared with control.

* NA indicates that values could not be calculated because oxidation was completely inhibited during 8 h of incubation.
Fig. 3. Inhibition of cell-mediated oxidation of LDL by 50 μmol/L melatonin and α-tocopherol.

LDL at a concentration of 100 mg/L protein was incubated with human monocyte-derived macrophages in Ham’s F-10 medium for 18 h at 37 °C. Cell-mediated oxidation was calculated as the difference between total TBARSs in the extracellular medium minus TBARSs in cell-free conditions. Values are means ± SD for triplicate measurements. Results are representative of three experiments using different preparations of LDL. (*), P < 0.001 vs control or melatonin; (#), P = 0.039 vs control.

**Discussion**

Melatonin has been suggested to have potent antioxidant properties that may prevent the development of cancer, atherosclerosis, and other consequences of aging (5, 10–13); however, these hypothetical effects are unproven (14, 15). In some animal studies, melatonin has been shown to have antioxidant properties in vivo, but often only at very high parenteral doses, e.g., 10 to 450 mg/kg body weight (5, 9, 10). In one small human study, nocturnal secretion of melatonin was decreased in 15 patients with coronary atherosclerosis (42); however, these data are insufficient to allow conclusions about the relationship between melatonin, antioxidant activity, and vascular disease (43). Thus, conclusive studies regarding the relevance of antioxidant properties of melatonin in prevention of disease are not available.

Because oxidation of LDL is believed to play an important etiologic role in the development of atherosclerosis, the capacity of melatonin to inhibit oxidation of LDL was tested in a standardized in vitro system. The susceptibility of LDL to undergo oxidation in this assay has been correlated with the severity of atherosclerosis in men with myocardial infarction (28). Although the results of other studies have suggested that high concentrations of melatonin may inhibit LDL oxidation (44–47), dose–response data comparing the capacity of melatonin to inhibit LDL oxidation with those of α-tocopherol and other antioxidants have been limited.

Melatonin had no antioxidant activity at physiologic concentrations and only moderate antioxidant activity at concentrations that were 4–6 orders of magnitude greater than peak physiologic concentrations and 50- to 100-fold higher than maximal serum concentrations achievable after large oral doses of melatonin up to 240 mg (39, 40). Very large doses of melatonin >1000 mg might achieve transient serum concentrations >5 μmol/L; however, the safety and clinical relevance of such doses are unclear. Although the indole moiety in melatonin has been suggested to be responsible for antioxidant activity (5), serotonin and tryptophan differed substantially from melatonin in their capacity to inhibit LDL oxidation. Physiologic concentrations of tryptophan (25–125 μmol/L) significantly inhibited LDL oxidation, whereas physiologic concentrations of serotonin (0.45–1.20 μmol/L) were inactive.

Moreover, at a concentration of 5 μmol/L, serotonin appeared to have prooxidant activity, producing accelerated oxidation of LDL. Halliwell and co-workers (48) also showed that serotonin was strongly prooxidant in an Fe(3+)-EDTA H2O-deoxyribose system. In other studies, 25 μmol/L serotonin stimulated uptake of oxidized LDL by macrophages (49). The biological importance of these findings is uncertain; however, these data suggest that supraphysiologic concentrations of serotonin may have the potential to enhance oxidation under some conditions. Both tryptophan and serotonin were more potent than melatonin at a concentration of 50 μmol/L.

α-Tocopherol has previously been demonstrated to be a potent inhibitor of LDL oxidation (34, 41, 50) and is hypothesized to protect against the development of atherosclerosis (19, 21, 22, 25, 26). In one recent double-blind placebo-controlled clinical trial, supplementation with α-tocopherol reduced the risk of cardiovascular death and nonfatal myocardial infarction by 47% (27). In the present study, α-tocopherol clearly was the most potent antioxi-
and was ~50-fold more efficacious than melatonin. Moreover, α-tocopherol had significant antioxidant activity at concentrations that were comparable to physiologic serum concentrations. Ascorbic acid, a water-soluble chain-breaking antioxidant, also was more potent than melatonin at all concentrations, and had significant antioxidant activity at physiologic concentrations.

The results of recent studies also demonstrated that melatonin did not substantially inhibit oxidation of LDL at concentrations <10–20 μmol/L (44–47). Moreover, a comparable dose–response relationship was demonstrated when the antioxidant activity of melatonin was tested in a specific radical-scavenging system using the radical-trapping reagent 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (8). In those studies, melatonin appeared to have greater radical-scavenging activity than tryptophan or ascorbic acid; however, it nonetheless had minimal scavenging activity at concentrations <50 μmol/L (8). Maximal radical-scavenging activity of melatonin in those studies occurred at concentrations of 150–200 μmol/L (8). Thus, melatonin appears to have substantial antioxidant activity only at markedly supraphysiologic concentrations.

In summary, extremely supraphysiologic concentrations of melatonin had weak antioxidant properties in this study; however, physiologic concentrations of α-tocopherol and ascorbic acid were significantly more efficacious at equimolar concentrations. Similarly, tryptophan and serotonin were significantly more potent than melatonin at the highest concentrations. Although high doses of melatonin (e.g., 10–450 mg/kg body weight parenterally) have been shown to have antioxidant properties in experimental animals (5–9), there currently are no data in animals or humans that conclusively demonstrate that melatonin plays a role in prevention of atherosclerosis (14, 15). These results suggest that the potential biological relevance of antioxidant properties of melatonin is uncertain and needs to be interpreted with caution until definitive studies are completed.

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References

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