Antioxidant role of melatonin in lipid peroxidation of human LDL

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Abstract The antioxidant effect of melatonin on LDL oxidation was studied in vitro using either a thermolabile initiator or copper ions to induce lipid peroxidation. Loading of LDL with melatonin showed only weak protection against oxidative damage as compared to α-tocopherol. In the presence of high concentrations of melatonin (1000 mol/mol LDL) in the medium a clear protective effect was found during lag- and propagation phase, albeit weaker than after loading with α-tocopherol. It is concluded that melatonin is not incorporated into LDL in sufficient concentrations to prevent lipid peroxidation effectively. When melatonin is present in the incubation medium during oxidation, a partitioning equilibrium between aqueous and lipid phase is established. Only under these conditions can melatonin act as a chain breaking antioxidant. The concentrations required, however, are far beyond those found in human plasma. Therefore, the data in this study do not support a direct physiological relevance of melatonin as an antioxidant in lipid peroxidation processes.

Key words: Low-density lipoprotein (LDL); Antioxidant; Lipid peroxidation; Melatonin; Free radical

1. Introduction

Cumulative neuronal insults associated with free radical production have been associated with the process of aging. The pineal hormone melatonin exhibits some radical scavenging effects [1], and its secretion declines progressively with age [2] leading to the hypothesis that it may protect against age-related processes, in part by attenuating the effects of free radical-induced neuronal damage [3].

More recently, several effects of melatonin were attributed to its antioxidative properties: cataract induction in rats as a consequence of free radical attack on lenticular macromolecules could be prevented by in vivo melatonin treatment [4], melatonin significantly decreased the frequency of radiation-induced chromosomal damage in lymphocytes [5,6], and a concentration-dependent protection by melatonin against in vitro kainic acid-induced oxidative damage was demonstrated in different rat brain regions [7–10].

Moreover, it was shown that melatonin is a potent radical scavenger, both for hydroxyl [11] and peroxyl radicals [12]. There are studies investigating the antioxidant activity of melatonin in lipid model systems [13] and also in human low-density lipoprotein (LDL) [14]. The results of the latter indicate that melatonin is able to scavenge the lipid peroxyl radicals which propagate the chain reaction of lipid peroxidation. These studies, however, leave some doubts as to the extent of the reported radical scavenging potential of melatonin: the concentrations of melatonin required were rather high and the effects comparatively small.

Preliminary data showed an association between impaired nocturnal secretion of melatonin and coronary artery disease ([15], Sakotnik et al., submitted). Hence, in the present study we examine the question, whether melatonin can protect human LDL against free radical induced lipid peroxidation in vitro, the characteristics of which have been extensively investigated (for a review, see [17]).

2. Materials and methods

AAPH (2,2′-azobis(amidinopropane hydrochloride)) was purchased from PolySciences Inc. (Warrington, USA), RRR-α-tocopherol from Henkel. All other chemicals used were purchased from Merck or Sigma (Vienna, Austria) and were of analytical grade, or better.

LDL was prepared from pooled EDTA plasma by ultracentrifugation using a single-step discontinuous gradient in a Beckman NVT65 rotor, as described previously [17]. LDL concentration was determined from its cholesterol content, using the CHOD-PAP enzymatic test kit (Boehringer-Mannheim, Germany), assuming a molar mass of 2.5 MDa and a cholesterol content of 32.2 weight %.

2.1. Loading of LDL with antioxidants

Pooled EDTA plasma was incubated with ethanolic melatonin (250 mM, 1% of total plasma volume) at 37°C under argon in the dark and LDL was subsequently isolated with the technique described above. As a positive control an ethanolic solution of RRR-α-tocopherol (250 mM, 1% of total plasma volume) was used, and as a negative control the same amount of pure ethanol [18].

2.2. Direct addition of melatonin to LDL

An ethanolic solution of melatonin was added to the oxidation mixture to give a total ethanol concentration of 0.5% (v/v). In blanks the same amount of pure ethanol was used.

2.3. Induction of LDL lipid peroxidation

2.3.1. AAPH-mediated LDL oxidation. LDL oxidation was performed at 37°C by addition of an aqueous solution of AAPH to a solution of 2 or 1 mg/mL LDL (total mass) to give a final AAPH-concentration of 1 mM. Decomposition of AAPH is slow enough to ensure a constant rate of peroxyl radical formation for several hours. Because both melatonin and AAPH exhibit considerable UV-absorption, which would have interfered with monitoring CD formation, we monitored oxygen consumption instead, using a Clark-electrode (Oxygraph, A. Paar, Austria). Oxygen consumption of blank AAPH solutions was subtracted. The effect of melatonin alone on oxygen consumption was negligible.

2.3.2. Copper-mediated LDL oxidation. Prior to oxidation, EDTA and KBr were removed by gel-filtration as described [19]. LDL oxi-

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Abbreviations: LDL, low-density lipoprotein; AAPH, (2,2′-azobis (amidinopropane hydrochloride)); BCSA, bathocuproine disulfonic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; CD, conjugated dienes; DPPH, 1,1-diphenyl-2-picryl-hydrazyl

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dation was performed at 37°C by addition of a 100 μM solution of $CuSO_4$ to a 0.1 μM solution of LDL in PBS (160 mM NaCl, 10 mM Na-phosphate, pH = 7.4), to give a final concentration of 1.6 μM. Progress of oxidation was monitored by following the formation of conjugated dienes (CD) spectrophotometrically (Beckman DU640 spectrophotometer, equipped with Peltier-thermostatted six-cell holder) at a wavelength of 234 nm [19].

2.3.3. Radical scavenging with DPPH (1,1-diphenly-2-picrylhydrazyl). The ability of melatonin to scavenge free radicals was tested with the stable free radical DPPH [20]. To an ethanolic solution of DPPH (0.2 mg/mL) 100 μM melatonin or tocopherol were added and the formation of the reduced form of DPPH was monitored at 517 nm in the spectrophotometer.

2.4. Determination of melatonin in LDL after incubation and reisolation

To 0.5 mg/mL LDL in PBS 50 μL EDTA (100 mg/mL) and 1 mL ethanol (containing 1 mg/mL BHT) were added. After extraction with 2 mL hexane, 1.2 mL of the hexane phase were evaporated in vacuo. The residue was dissolved in the eluent for HPLC, and chromatographed according to [21], using fluorescence detection at 290 nm/357 nm (ex/em) with external standardization.

2.5. Determination of copper-reduction with bathocuproine disulfonic acid

Reduction of Cu^{2+} by tocopherol and melatonin was monitored in 50 vol. % ethanol with bathocuproine disulfonic acid (BCSA, Sigma-Aldrich, Vienna, Austria). BCSA selectively chelates Cu^{+} ions by formation of a colored, redox-inactive complex, $Cu(I)(BCSA)_2$ ($\varepsilon_{480} = 13\,500\ M^{-1}\ cm^{-1}$ [22]). The test contained 50 μM of α-tocopherol, melatonin or both. BCSA concentration was 500 μM and the reaction was started by adding 100 μM $CuSO_4$.

2.5.1. Determination of lag-time and propagation rate. Lag-time was determined as the time-coordinate of the intersection of the tangents to the increase in CD or decrease of oxygen concentration during lag- and propagation phase, as described [19]. Propagation rate is defined as the (maximal) rate of formation of CD or consumption of oxygen, respectively.

3. Results

Loading of LDL with α-tocopherol is an efficient means to increase the content of this endogenous, lipophilic antioxidant, which consequently leads to a significant prolongation of the lag-phase [18,20,24]. Because of the reported lipophilicity of melatonin [25] we attempted to load LDL with melatonin by the same procedure.

We investigated the effect of melatonin on AAPH-induced LDL oxidation: AAPH initiates lipid peroxidation by decomposing thermally into two carbon-centered radicals which are subsequently rapidly oxygenated to form the initiating peroxyl radicals. Fig. 1 shows the changes in lag-time and propagation rates, obtained from oxygen consumption, both in the case of pre-incubation of LDL with melatonin and tocopherol, as well as the effect of direct addition to the oxidation mixture. Pre-incubation with melatonin increased lag-time only marginally by 11% (total lag-time 39 min) and did not significantly reduce propagation rate. In contrast, loading with α-tocopherol as a positive control prolonged lag-time by 49% (total lag-time 52 min). The control showed a lag-time of 35 min and maximum oxygen consumption of 21.7 nM s^{-1} – the difference compared to the control in the pre-incubation experiment is due to the presence of ethanol: in the preincubation experiment practically no ethanol should be present during oxidation, as it is removed during LDL isolation. In the case of direct addition, the control (0.5% ethanol) showed a lag-phase of 51 min and maximum oxygen consumption of 54.9 nM s^{-1}. Increasing concentrations of melatonin did not significantly change lag-time, but melatonin at 25 μM decreased the rate of propagation to 60% of the control (33.0 nM s^{-1}). While the effect of tocopherol is limited to the lag-phase exclusively, the effects of melatonin can be observed during both lag- and propagation-phase.

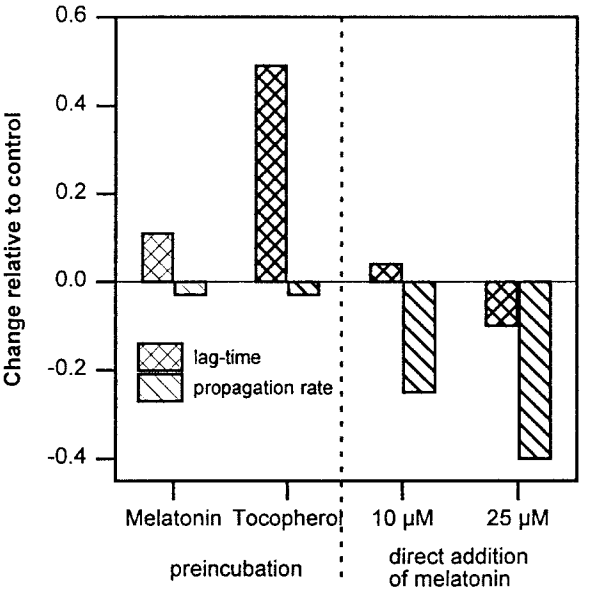

Fig. 1. Relative changes in lag-time and propagation rate for AAPH-mediated oxidation in the presence of melatonin and α-tocopherol. A 0.1 μM solution of LDL in PBS was oxidized with 1 mM AAPH at 37°C. Oxidation was monitored by measuring oxygen consumption. Melatonin was either added at the indicated concentrations or LDL was loaded with either melatonin or α-tocopherol. For the controls, pure ethanol was added in the appropriate concentration either directly to the incubation or during preincubation of plasma. Blank oxygen consumption by AAPH was subtracted in every case.

Table 1
Effect of plasma preincubation with melatonin or tocopherol on LDL oxidation

	Lag-time (min)	% Change in lag-time
Control	80.8 ± 0.92	–
Melatonin	86.3 ± 1.96	6.75 ± 1.67
α-Tocopherol	128.8 ± 6.33	61.1 ± 7.43

Pooled plasma was preincubated with an ethanolic solution of either melatonin or α-tocopherol (250 mM, 1% v/v added). Controls were preincubated with the same amount of pure ethanol. Data are mean ± S.E.M. of 3–4 measurements.

In copper-mediated LDL oxidation, lipid peroxyl radicals are generated indirectly within the lipid phase [26]. Table 1 gives a summary of the effects of incubation with melatonin or tocopherol of Cu^{2+}-induced LDL oxidation, respectively. Fig. 2 shows one representative experiment: it is obvious that 'loading' with melatonin had no significant effect.

In contrast to this, direct addition of melatonin to the oxidation mixture significantly suppressed Cu^{2+}-mediated oxidation as shown in Fig. 3. Melatonin in 50 to 1000-fold molar excess (5–100 μM) increased lag-time in a concentration-dependent fashion, up to 20%. In addition, maximum rate of CD formation, as well as the total maximum amount of CD formed, decreased by up to 20% for melatonin concentrations of 50 μM or higher, also depending on concentration. Since 0.5% of ethanol itself produced a significant increase in lag-time and a decrease in propagation rate, care was taken to use

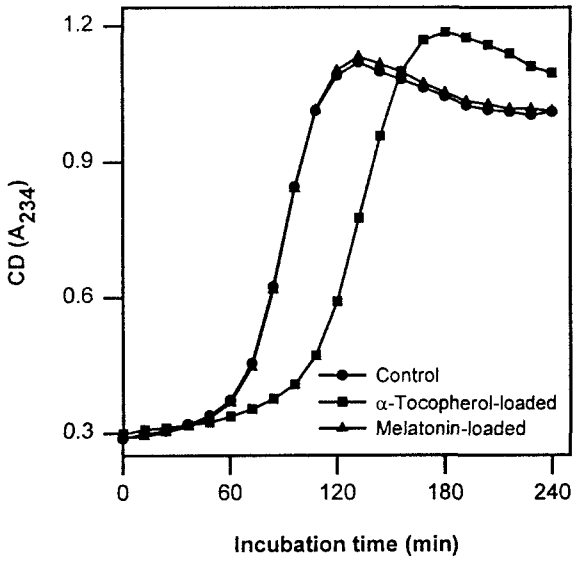

Fig. 2. Effect of plasma incubation with melatonin and α-tocopherol on copper-mediated LDL oxidation. Plasma was loaded with either melatonin or α-tocopherol or with pure ethanol as a control. After isolation, 0.1 μM LDL was oxidized with 1.6 μM $CuSO_4$, and formation of conjugated dienes recorded at 234 nm. A significant prolongation of lag-time was observed for LDL from plasma incubated with α-tocopherol while only a small effect was found after incubation with melatonin.

the same concentration of ethanol in all experiments, and therefore LDL oxidation in the presence of 0.5% ethanol was used as a blank. The same result as in Fig. 3 was obtained in experiments where the ethanol concentration was only 0.05%, thus excluding a direct melatonin-solubilizing effect of ethanol in LDL (not shown).

These results indicate that much less melatonin might be incorporated into LDL than expected. Consequently, melatonin concentration in 'melatonin-loaded' LDL, as estimated by HPLC, was below 0.05 mol melatonin per mol LDL (data not shown).

To exclude the possibility of a redox-inactivation of copper (e.g. by chelation) by melatonin, we compared the reduction of copper by tocopherol and melatonin in presence of BCSA, a selective chelator for Cu^+, in 50% ethanol. Melatonin is a weak reductant for copper – it took 15 min to reduce 50 μM Cu^{2+} under our assay conditions (50 μM reductant, 100 μM Cu^{2+}). Tocopherol alone reduced copper very rapidly – within 10 s required for addition of copper, reduction was complete. Reduction of copper by 50 μM tocopherol in presence of 50 μM melatonin was equally rapid, indicating that no redox-inactivation of copper by melatonin occurs.

Compared to α-tocopherol, the radical scavenging potential of melatonin, tested by its ability to scavenge DPPH radicals, is negligible: while DPPH was scavenged completely by α-tocopherol, no significant consumption of DPPH by melatonin was observed (Fig. 4).

4. Discussion

Lipid peroxidation in LDL performed under the conditions described, usually exhibits three phases: (i) a lag-phase with very slow formation of conjugated dienes (CD), during which endogenous lipophilic antioxidants such as α-tocopherol prevent radical chain propagation. After consumption of these endogenous antioxidants, (ii) chain propagation starts which is accompanied by a rapid increase in CD formation. Later in the process, (iii) CD are destroyed in subsequent reactions [24]. The length of the lag-phase represents an accepted measure for the resistance of LDL to oxidation and thus the effect of endogenous antioxidants. In contrast to endogenous antioxidants, amphiphilic antioxidants from the aqueous phase can extend their effect to the propagation phase, i.e. they will lower the rate of propagation.

Pre-incubation with α-tocopherol increases its content in LDL by a factor of two and more [18,23,24], which results in a considerable increase of lag-time. In contrast, attempts to load LDL with melatonin had almost no effect, both in AAPH- and Cu^{2+}-mediated oxidation, even though melatonin has been reported to be both lipophilic and a potent radical scavenger. Determination of melatonin in LDL after loading showed only minute quantities present excluding accumulation and persistence of melatonin in the lipid phase of LDL.

In contrast, direct addition of melatonin to the oxidation assay, at up to 1000-fold excess over LDL, led to a significant antioxidative effect comparable to the results of others [14], i.e. lag-times were prolonged, and propagation rates were lower. However, our data show that melatonin acts over the entire oxidation time, on both AAPH- and Cu^{2+}-mediated oxidation, and not predominantly during the lag-phase, when endogenous antioxidants of LDL, such as α-tocopherol, are effective. Thus, at the end of the lag-phase, when highly potent endogenous antioxidants of LDL are already consumed, melatonin still has a retarding effect.

In case of AAPH-mediated oxidation, melatonin might scavenge AAPH-derived peroxyl radicals directly in the aqueous medium, thus reducing rate of initiation. However, a similar effect of melatonin is also observable in copper-mediated oxidation, where lipid peroxyl radicals are generated indirectly

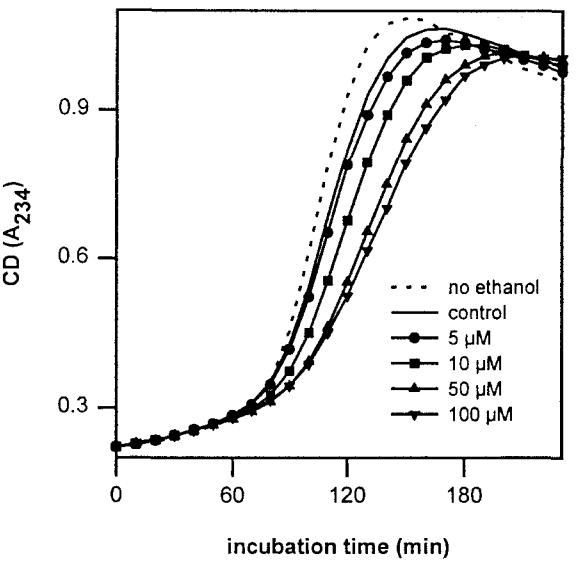

Fig. 3. Effect of direct addition of melatonin on copper-mediated LDL oxidation. The indicated concentration of melatonin was added to a solution of 0.1 μM LDL in PBS and oxidation started by addition of 1.6 μM $CuSO_4$. Oxidation was monitored by the absorption of conjugated dienes at 234 nm. Addition of increasing melatonin concentrations led to a prolongation of the lag-phase and a reduction of maximum rate of formation of conjugated dienes.

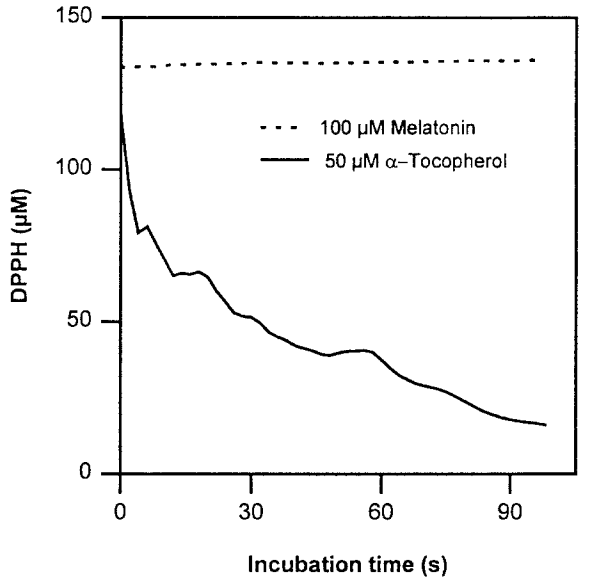

Fig. 4. Scavenging of the stable free radical DPPH by α-tocopherol and melatonin. To a 100 μM ethanolic solution of DPPH, 100 μM melatonin or 50 μM α-tocopherol were added and the decrease of DPPH absorption was monitored at 517 nm every 2 s.

from a series of redox reactions [26] within the lipid phase. Since a retarding effect of melatonin due to redox inactivation of copper can be excluded from the result of competitive copper reduction by melatonin and tocopherol, melatonin must enter the lipid phase of LDL to prevent oxidation in this case. Since using 10 times less ethanol in the assay led to essentially the same effect, the possibility can be ruled out that ethanol (0.5 vol% used for disperging melatonin into the aqueous solution) might increase melatonin solubility in LDL.

Our results therefore indicate a continuous exchange of melatonin between the aqueous environment and the LDL lipid phase during the whole oxidation period with incorporation of very low quantities into LDL. Melatonin thus could act as an amphiphilic chain breaking antioxidant in a lipid environment, by scavenging peroxyl radicals, either derived directly from AAPH, or indirectly from copper.This is supported by recent findings which indicate a considerable solubility in aqueous medium in the millimolar range [27].

Our observation that melatonin, compared to tocopherol, is rather unreactive against DPPH (Fig. 4), confirms a recent report that melatonin cannot scavenge radicals of low reactivity (such as superoxide or, in this work, DPPH), but will react easily with more reactive species [13], such as lipid peroxyl or alkoxyl radicals. Thus melatonin is obviously a less potent and less lipophilic antioxidant than α-tocopherol, albeit it exerts its effects in the lipid phase of LDL, resulting in moderate prolongation of the lag-phase and decrease of propagation rate. In the paper cited above it is speculated that melatonin might change the properties of the outer phospholipid layer of LDL thereby reducing the overall mobility of the reactants or the accessibility of lipids to initiators of lipid peroxidation. Although this is in line with our observation that the effect of melatonin is not limited to the lag-phase, our finding that the LDL molecule cannot be loaded with melatonin in significant quantities contradicts this hypothesis. It is most unlikely that incorporation of much less than one melatonin molecule, among several hundreds of other molecules that constitute the LDL surface [16] is sufficient for a distinct change of the surface properties of all LDL particles. Furthermore, a structural effect of melatonin would equally affect all phases of oxidation, or even dominate during the lag-phase. However, we show that the effect of melatonin addition is most prominent during propagation but small during the lag phase. The moderate reactivity against radicals and the low melatonin concentration and persistence in LDL fully explain the effects observed.

Based on our results a more differentiated concept of the antioxidant effect of melatonin is proposed: the assumed lipophilicity of melatonin is not high enough to permit accumulation in the lipid phase without constant supply from surrounding bulk aqueous solution. Moreover, its antioxidant activity is considerably lower than that of α-tocopherol. Given the concentrations of melatonin in aqueous medium required to exhibit a significant protection of LDL against lipid peroxidation as compared to the in vivo situation, we exclude a significant modulating role of endocrine melatonin as an antioxidant for in vivo lipid peroxidation processes. However, an indirect role via regulation of other antioxidant defense systems by melatonin [13,28,29] cannot be ruled out.

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