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Melatonin prevents changes in microsomal membrane fluidity during induced lipid peroxidation

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Abstract We tested the effect of melatonin on membrane fluidity in microsomes of a rat liver model in which lipid peroxidation was induced by the addition of $FeCl_3$, ADP and NADPH. Membrane fluidity was monitored using fluorescence spectroscopy and lipid peroxidation was estimated by quantifying malonaldehyde (MDA)+4-hydroxyalkenals (4-HDA) concentrations following the induction of lipid peroxidation with and without pre-incubation with melatonin (1 μM–3 mM). Membrane rigidity increased during induced lipid peroxidation while melatonin reduced in a concentration-dependent manner both membrane rigidity and MDA+4-HDA generation. Melatonin's protective effect may relate to its known ability to scavenge free radicals and function as an antioxidant.

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Key words: Melatonin; Antioxidant; Free radical; Membrane fluidity; Lipid peroxidation; Microsome

1. Introduction

Lipid peroxidation is an autooxidative process initiated by a variety of free radicals to which polyunsaturated fatty acids present in cell membranes are susceptible [1]; the process of lipid peroxidation leads to the formation of LOO·, endoperoxides and hydroperoxides [2]. Once formed, unstable LOO· are able to propagate a chain reaction of the lipid peroxidation process [3]. Associated with these changes in the membrane, several authors claim an increase in phospholipid bilayer rigidity after lipid peroxidation [4,5]. The physiological importance of modifications in the physical properties of membranes resides in their relation to numerous cellular functions, including the activity of membrane-associated enzymes solute transport and hormone-induced signal transduction processes [2,6]. Both, lipid peroxidation and membrane fluidity have been implicated in disease processes and aging pathophysiology [7–9]. Several agents are known to be stabilizers or protectors of cell and organelle membranes because of their inhibitory effects on lipid peroxidation [5,10].

N-Acetyl-5-methoxytryptamine or melatonin is produced by the pineal gland in all vertebrates. This chemical mediator modulates a variety of endocrine, neural and immune functions [11,12]. Recently, its direct scavenging action against free radicals was reported [13]. Besides its ability to scavenge highly reactive radicals, melatonin's antioxidant activity is augmented by its ability to stimulate enzymes related to the antioxidative defense system [14–17]. In addition, the efficacy of melatonin as an antioxidant may relate to the ease with which it crosses biological membranes [18]. In this report we investigated, utilizing hepatic microsomal membranes, the effect of melatonin on membrane fluidity that was modulated by lipid peroxidation due to Fe^{3+}/ADP/NADPH.

2. Materials and methods

Melatonin, ADP, ferric chloride, NADPH and EDTA were purchased from Sigma (St. Louis, MO). TMA-DPH was purchased from Molecular Probes (Eugene, OR). The Bioxytech LPO-586 kit for lipid peroxidation was obtained from Caymen Chemical (Ann Arbor, MI). Other chemicals used were of analytical grade and came from commercial sources. Melatonin was diluted in ethanol and water and TMA-DPH in THF and water. Ethanol and THF concentrations in the incubation volume were 2 and 0.4%, respectively. Other chemicals were diluted in the incubation buffer.

Male Sprague-Dawley rats, weighing 230–250 g, were sacrificed by decapitation and the livers were removed quickly, washed in 0.9% NaCl (4°C), frozen in liquid nitrogen and stored at −80°C prior to use. Pooled microsomes were isolated according Yu et al. [19]. Briefly, liver was homogenized 1 : 10 w/v in 140 mM KCl/20 mM HEPES buffer (pH 7.4) and the resulting suspension centrifuged at $1000\times g$ for 10 min at 4°C. The supernatant was centrifuged at $105\,000\times g$ for 60 min at 4°C. The pellet obtained was re-suspended in the buffer and centrifuged at $10\,000\times g$ for 15 min at 4°C. The supernatant, which contained only microsomal fraction, was centrifuged once at $105\,000\times g$ for 60 min at 4°C. The final pellet was re-suspended 1 : 1 w/v and kept at −80°C until assay.

Microsomes (0.5 mg/ml microsomal protein), suspended in 50 mM Tris-HCl buffer (pH 7.4), were incubated at 37°C for 30 min with melatonin (3 mM, 1 mM, 300 μM, 100 μM, 10 μM or 1 μM). Lipid peroxidation was induced in the membrane samples by adding $FeCl_3$ (0.2 mM), ADP (1.7 mM), NADPH (0.2 mM) and the incubation was carried out at 37°C for 20 min with shaking. The reaction was stopped by adding EDTA (2 mM). Kinetic studies were performed to determine the optimal incubation times. Control microsomes and those with induced lipid peroxidation were treated under the same conditions (incubation and addition of ethanol) as samples treated with melatonin. In another study, only microsomes incubated with similar concentrations of melatonin and controls were used. Solutions of melatonin, $FeCl_3$, ADP and NADPH were prepared fresh just prior to use.

Membrane fluidity and MDA+4-HDA were measured after incubations were complete. Fluidity was studied using the TMA-DPH as probe. Its incorporation into microsomal membrane and the determination of membrane fluidity was done according to the method of Yu et al. [19]. Microsomes (0.5 mg protein) were re-suspended in Tris (3 ml final volume) and incorporated with TMA-DPH (66.7 nM). After mixing vigorously on a vortex for 1 min, the preparation was incubated for 30 min at 37°C. Fluorescence measurements were carried in a Perkin-Elmer LS-50 Luminescence Spectrometer equipped with a circulatory water bath to maintain the temperature of 22 ± 0.1°C.

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Abbreviations: MDA, malonaldehyde; 4-HDA, 4-hydroxyalkenals; LOO·, peroxyl radical; ·OH, hydroxyl radical; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene-sulfonate; THF, tetrahydrofuran

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Excitation and emission wavelengths of 360 nm and 430 nm were used, respectively. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel (I_{V_V}) or perpendicular (I_{V_H}) to the excitation plane. A correction factor for the optical system, G, was used. Polarization (P) and anisotropy (A) were calculated by equations 1 and 2, respectively.

$$P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}} \quad (1)$$

$$A = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + 2GI_{V_H}} \quad (2)$$

An inverse relationship exists between membrane fluidity and polarization and anisotropy [19]; thus, membrane fluidity is expressed as 1/P and 1/A, respectively. Membrane fluidity was calculated from triplicate determinations. Combined MDA+4-HDA concentrations are used as an index of lipid peroxidation of membranes [20]; this was measured by a colorimetric assay using the kit mentioned above. Results are presented as nmol MDA+4-HDA/mg microsomal protein. Protein concentration was determined by the Bradford method [21] using bovine serum albumin as standard.

The data are expressed as arithmetic mean and S.E. values. Mean differences were determined using a paired *t*-test, with a level of significance $P < 0.05$.

3. Results

Under the conditions of this study, the addition of $FeCl_3$, ADP and NADPH decreased microsomal membrane fluidity, quantitated as changes in 1/P and 1/A, and increased MDA+4-HDA levels. Pre-incubation of the microsomes with melatonin at the concentration of 300 μM or greater increased significantly ($P < 0.05$) membrane fluidity and reduced MDA+4-HDA formation compared to those in the non-melatonin-treated samples. Melatonin at 3 mM prevented membrane rigidity completely; in this case no significant differences in 1/P and 1/A were observed relative to controls. Results of 1/P, 1/A and MDA+4-HDA measurements after various treatments are summarized in Table 1. The ability of melatonin to inhibit membrane fluidity and to decrease MDA+4-HDA was dose-dependent. The concentration of melatonin required to inhibit MDA+4-HDA formation by 50% (1.25 mM), i.e. the IC_{50}, was slightly less than that required to prevent membrane rigidity (1.5 mM). IC_{50} values were calculated from the data presented in Fig. 1.

In a subsequent experiment, to clarify whether melatonin may alter membrane fluidity in the absence of lipid peroxidation, microsomes were incubated with the same concentrations of melatonin used in the initial studies. No significant differences in membrane fluidity or MDA+4-HDA levels were found between control samples and those incubated with melatonin. Table 2 summarizes these results.

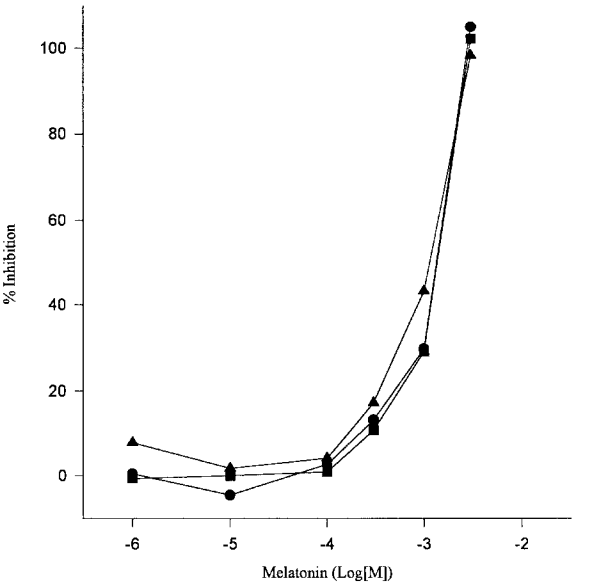

Fig. 1. Ability of melatonin to reduce microsomal membrane rigidity and to inhibit lipid peroxidation. (●) 1/Polarization; (■) 1/Anisotropy; (▲) MDA+4-HDA. Melatonin is expressed as a logarithm of its molar concentration.

4. Discussion

Biophysical measurements of membrane fluidity provide a structural alteration of the consequences of lipid peroxidation. Lipid peroxidative stress is known to decrease membrane fluidity in microsomes [22] and other cellular membranes [23,24]. The present results, using TMA-DPH as a fluorescence probe, are consistent with these observations. Structural changes in the membrane including the loss of polyunsaturated fatty acids [2,25] and the formation of cross-linking of lipid–lipid and lipid–protein moieties [5,25,26] are known to be the basic mechanisms for membrane rigidity.

In the present studies, when microsomes were incubated with different concentrations of melatonin prior to the induction of lipid peroxidative damage, a reduction in P and A parameters, and thus the increase of membrane fluidity was observed. A corresponding protective effect of melatonin also was observed as indicated by the extent of MDA+4-HDA generation.

Table 1
The effects of incubation with melatonin prior to induced lipid peroxidation on membrane fluidity (1/P and 1/A) and MDA+4-HDA concentrations

	1/P	1/A	MDA+4-HDA (nmol/mg protein)
Control	3.234 ± 0.020	4.330 ± 0.028	2.872 ± 0.561
Induced lipid peroxidation	2.796 ± 0.016[a]	3.684 ± 0.024[a]	80.380 ± 3.237[a]
Induced lipid peroxidation+melatonin			
(3 mM)	3.256 ± 0.016[b]	4.344 ± 0.024[b]	4.274 ± 0.498[ab]
(1 mM)	2.926 ± 0.020[ab]	3.872 ± 0.032[ab]	46.940 ± 2.249[ab]
(300 μM)	2.854 ± 0.007[ab]	3.754 ± 0.024[ab]	67.020 ± 3.205[ab]
(100 μM)	2.808 ± 0.010[a]	3.690 ± 0.023[a]	77.140 ± 3.758[a]
(10 μM)	2.776 ± 0.009[a]	3.684 ± 0.010[a]	78.980 ± 3.270[a]
(1 μM)	2.798 ± 0.009[a]	3.680 ± 0.018[a]	74.220 ± 3.803[ab]

Data are expressed as mean ± S.E. values obtained in five independent experiments.
[a]Denotes statistical differences ($P < 0.05$) vs. control and [b]vs. lipid peroxidation.

Table 2
MDA + 4-HDA concentrations and membrane fluidity, expressed as an inverse of polarization or anisotropy after incubation with melatonin

	1/P	1/A	MDA + 4-HDA (nmol/mg protein)
Control	3.185 ± 0.018	4.262 ± 0.035	3.290 ± 0302
Melatonin			
(3 mM)	3.197 ± 0.008	4.293 ± 0.019	3.170 ± 0.365
(1 mM)	3.168 ± 0.016	4.235 ± 0.021	3.235 ± 0.236
(300 μM)	3.185 ± 0.029	4.302 ± 0.059	3.430 ± 0.433
(100 μM)	3.152 ± 0.020	4.200 ± 0.024	3.370 ± 0.311
(10 μM)	3.150 ± 0.011	4.225 ± 0.010	3.385 ± 0.355
(1 μM)	3.153 ± 0.017	4.267 ± 0.028	3.360 ± 0.312

Results are mean ± S.E. values obtained in four experiments.

Initiation and propagation of lipid peroxidation in microsomes is a complex chain reaction wherein the ·OH, LOO· and perferryl ion are involved [27] in a process dependent on NADPH, ADP, Fe^{3+} cytochrome P_{450} [28]. Melatonin's ability to reduce free radical-mediated damage is documented in a variety of investigations in which both in vivo and in vitro models of free radical generation were used [29,30]. Its role as a scavenger against the highly reactive ·OH [13], a process that involves electron donation [31], and also its efficacy in preventing damage due to the LOO·, have been demonstrated [32]. The close relationship between biochemical membrane damage, expressed by MDA+4-HDA formation, and the decrease in membrane fluidity (Fig. 1), which were both inhibited by melatonin, make likely a scavenging mechanism to explain the reduction in membrane rigidity. Moreover, the lack of significant changes in polarization and anisotropy in the experiment performed to investigate whether melatonin would modify membrane fluidity in the absence of lipid peroxidation re-inforces the hypotheses that free radical scavenging may be the underlying mechanism whereby melatonin promotes membrane stabilization.

Other antioxidants also are believed to prevent changes in membrane fluidity induced by peroxidative damage by reducing the generation of free radicals [10,22,23]. Some of them, however, only partially reduce the changes or even decreased membrane fluidity [33].

A particular interest is that melatonin only changed membrane fluidity when it accompanied oxidative stress. This phenomenon may become significant in understanding the effect of the reported antiaging action of melatonin. During the aging process it is known that membrane rigidity increases as a consequence of lipid peroxidation [19]. The present results indicate that melatonin's action is to stabilize membrane fluidity presumably at the optimal level. There are some scavengers which decrease membrane fluidity under these circumstances [34,35].

Evidence to date suggests that melatonin resists oxidative stress by scavenging free radicals [13,29–32] and by stimulating the activity of certain antioxidative enzymes [14–17]. The current findings show that stabilizing cell membranes may be another important function by which melatonin reduces oxidative damage to cells and organs. Alterations in membrane fluidity have major consequences in terms of cellular function [6] and, thus, melatonin's ability to stabilize membranes may further contribute to the cell protective actions of this molecule [36].

In conclusion, these results indicate that melatonin stabilizes microsomal membranes and resists the rigidity induced by free radical attack. Based on these results and those from previous studies [37], it is presumed that melatonin's ability to stabilize cellular membranes is a result of its free radical scavenging activity and is related to its ability to reduce lipid peroxidation.

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