Melatonin Enhances Tamoxifen's Ability to Prevent the Reduction in Microsomal Membrane Fluidity Induced by Lipid Peroxidation

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Abstract. The indoleamine melatonin and the synthetic antiestrogenic drug tamoxifen seem to have similar mechanisms in inhibiting the growth of estrogen receptor positive breast cancer cells. In this study, we compared the ability of these molecules, alone and in combination, in stabilizing microsomal membranes against free radical attack. Hepatic microsomes were obtained from male rats and incubated with or without tamoxifen (50–200 μM), melatonin (1 mM) or both; lipid peroxidation was induced by addition of $FeCl_3$, NADPH and ADP. After oxidative damage, membrane fluidity, measured by fluorescence polarization techniques, decreased whereas malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations increased. Incubation of the microsomes with tamoxifen prior to exposure to free radical generating processes inhibited, in a dose-dependent manner, the increase in membrane rigidity and the rise in MDA+4-HDA levels. When melatonin was added, the efficacy of tamoxifen in preventing membrane rigidity was enhanced. Thus, the IC_{50}s for preventing membrane rigidity and for inhibiting lipid peroxidation obtained for tamoxifen in the presence of melatonin were lower than those obtained with tamoxifen alone. Moreover, tamoxifen (50–200 μM) in the presence of melatonin reduced basal membrane fluidity and MDA+4-HDA levels in microsomes. These synergistic effects of tamoxifen and melatonin in stabilizing biological membranes may be important in protecting membranes from free radical damage.

Key words: Melatonin — Tamoxifen — Antioxidant — Lipid peroxidation — Membrane fluidity — Microsome

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Introduction

Free radicals generated within subcellular compartments damage macromolecules which lead to structural changes and functional alterations of cellular organelles. A manifestation of free radical injury to biological membranes, i.e., lipid peroxidation, is an autooxidative chain reaction in which polyunsaturated fatty acids in the membrane are the substrates. Cancer, aging and several degenerative diseases have been related to free radical damage and lipid peroxidation [1, 22, 27, 34]. As a result, there is considerable interest in molecules that limit free radical damage including lipid peroxidation.

Tamoxifen is a antiestrogenic drug currently used for the treatment of breast cancer [40]. The mechanism by which it inhibits cell proliferation is known to be related to a competition with endogenously produced estrogens for binding to the estrogen receptor [10, 41]. Despite this proposed mechanism of action, epidemiological studies have shown improvements during treatment with tamoxifen occur in patients essentially devoid of estrogen receptors, i.e., negative or under 10 fmol receptor/mg protein in their breast tissue [23]. Because of this, several authors have suggested other potential mechanisms for tamoxifen that are unrelated to its binding to the estrogen receptor; these alternate mechanisms may involve tamoxifen's ability to regulate tumor growth factors [8, 12] and its antioxidant properties [19, 68, 69].

Melatonin is a chemical mediator produced mainly by the pineal gland of mammals. Classically, the functions of this indoleamine have been related to the modulation of neuroendocrine, circadian and immune functions [31, 52]. Previous workers have also shown, however, that melatonin has an oncostatic role in breast cancer [11, 16] and other tumors as well [5]. The addition of melatonin to culture medium inhibits the growth

of MCF-7 human breast cancer cells [35]. Melatonin delays the cell cycle [15], like tamoxifen [60], and regulates the action of growth factors on these cells [14, 46]. In recent years, melatonin's antioxidant activity has been revealed; this function of melatonin is likely at least in part receptor independent [61]. For example, melatonin has been shown to directly detoxify free radicals and to reduce oxidative damage to DNA and lipids under a number of experimental situations [53, 54]. Hence, it has been proposed that melatonin may play a role in protecting against free radical-related diseases.

Both tamoxifen and melatonin are known for their oncostatic actions and for their ability to inhibit lipid peroxidation. Their interaction, however, has not been investigated in terms of reducing oxidative damage and in preventing changes in membrane fluidity. In the present study, we tested the combination of tamoxifen and melatonin in hepatic microsomal membranes which were treated with $FeCl_3$, ADP and NADPH to induce lipid peroxidation. The changes in membrane fluidity were monitored by fluorescence spectroscopy and microsomal MDA+4-HDA concentrations were estimated as an index of lipid peroxidation.

Materials and Methods

CHEMICALS

Tamoxifen, $FeCl_3$, ADP, NADPH and EDTA were purchased from Sigma (St. Louis, MO), melatonin from Regis Technologies (Morton Grove, IL) and TMA-DPH from Molecular Probes (Eugene, OR). The Bioxytech LPO-586 kit for lipid peroxidation was obtained from Caymen Chemical (Ann Arbor, MI). Other chemicals utilized were of the highest analytical grade and came from commercial sources. Tamoxifen was diluted in absolute ethanol, melatonin in ethanol and distilled water and TMA-DPH in tetrahydrofuran and water. Ethanol and tetrahydrofuran were 3% and 0.4% in the final mixture, respectively. $FeCl_3$, ADP, NADPH and EDTA were diluted in the incubation buffer. Tamoxifen, melatonin, $FeCl_3$, ADP and NADPH solutions were prepared immediately before use.

ANIMALS AND PREPARATION OF MICROSOMES

Hepatic microsomes were obtained from male Sprague-Dawley rats weighing 230–270 g. Animals received standard food and water ad libitum. After being acclimated for one week, they were sacrificed by decapitation and the liver was removed quickly, washed in 0.9% NaCl (4°C), frozen in liquid nitrogen and stored at −80°C. Pooled microsomal membranes were isolated by the differential centrifugation technique described elsewhere [29].

INDUCTION OF LIPID PEROXIDATION

Aliquots of microsomal membranes (0.5 mg/ml microsomal protein) were suspended in 50 mM Tris-HCl buffer (pH = 7.4) and incubated with tamoxifen (50 μM, 100 μM, 150 μM, 175 μM and 200 μM) for 30 min at 37°C. Lipid peroxidation was initiated by addition of $FeCl_3$ (0.2

mM), ADP (1.7 mM) and NADPH (0.2 mM) and incubation in a water bath for 20 min at 37°C under aerobic conditions. The peroxidative reaction was stopped by addition of EDTA (2 mM). Control microsomes with and without induced lipid peroxidation were exposed to the same incubation conditions as those treated with tamoxifen.

In other experiments, melatonin (1 mM) was added at the same time as tamoxifen. In these studies, melatonin was also tested in the absence of tamoxifen. Identical procedures as in the previous experiments were followed, i.e., tamoxifen, $FeCl_3$, ADP and NADPH concentrations and incubations were the same.

MEASUREMENT OF MEMBRANE FLUIDITY AND MDA+4-HDA CONCENTRATIONS

Membrane fluidity, measured in triplicate, was performed according to method of Yu et al. [72]. The fluorescence probe used was TMA-DPH. Briefly, 0.5 mg microsomal protein were resuspended in buffer (3 ml final volume), vortexed for 1 min with the probe (66.7 nM) and incubated with shaking at 37°C for 30 min to ensure the uniform incorporation of the probe in the membranes. Polarization (P) and anisotropy (A) parameters (mean of 10 observations) were obtained using a Perkin-Elmer LS-50 Luminiscence Spectrometer equipped with a circulator bath to maintain the temperature of 22.0 ± 0.1°C during the assay. Excitation and emission wavelengths of 360 nm and 430 nm were used, respectively. Polarization and anisotropy were calculated using the Eqs. 1 and 2.

$$P = \frac{I_{V_V} - G I_{V_H}}{I_{V_V} + G I_{V_H}} \quad (1) \qquad A = \frac{I_{V_V} - G I_{V_H}}{I_{V_V} + 2 G I_{V_H}} \quad (2)$$

where I_{V_V} and I_{V_H} are the emission intensity of vertically polarized light detected by an analyzer oriented parallel or perpendicular, respectively, to the excitation plane and G is the correction factor for the optical system. Membrane fluidity was expressed as the inverse of P and A [72].

MDA+4-HDA concentrations are considered an index of lipid peroxidation in membranes [25]. These concentrations were determined by the colorimetric kit mentioned above. Microsomal protein was measured by the Bradford method [6] using bovine serum albumin as standard.

STATISTICAL ANALYSIS

Data obtained are expressed as arithmetic means and standard errors. An inferential statistical procedure was performed using the t-test. The level of significance was accepted with $P < 0.05$.

Results

The data relating to the ability of tamoxifen to reduce microsomal membrane rigidity and to inhibit MDA+4-HDA formation are summarized in Table 1. Incubation of microsomes with tamoxifen prior to induction of lipid peroxidation increased membrane fluidity in a concentration-dependent manner. Accompanying the membrane stabilizing action of tamoxifen, a progressive inhibition in MDA+4-HDA formation was noted. The concentrations of tamoxifen required to reduce the de-

Table 1. Membrane fluidity, $1/P$ and $1/A$, and MDA+4-HDA concentrations in microsomes treated with agents to induce lipid peroxidation without and with tamoxifen

	l/P	l/A	MDA+4-HDA (nmol/mg protein)
Control	3.20 ± 0.01	4.29 ± 0.02	2.84 ± 0.38
Induced lipid peroxidation	$2.76 \pm 0.01^{(a)}$	$3.69 \pm 0.02^{(a)}$	$87.45 \pm 3.19^{(a)}$
Induced lipid peroxidation + Tamoxifen			
(200 μM)	$3.13 \pm 0.01^{(a,b)}$	$4.21 \pm 0.02^{(a,b)}$	$8.11 \pm 1.45^{(a,b)}$
(175 μM)	$3.03 \pm 0.01^{(a,b)}$	$4.04 \pm 0.01^{(a,b)}$	$14.77 \pm 0.54^{(a,b)}$
(150 μM)	$2.85 \pm 0.02^{(a,b)}$	$3.78 \pm 0.03^{(a)}$	$29.30 \pm 2.51^{(a,b)}$
(100 μM)	$2.76 \pm 0.01^{(a)}$	$3.63 \pm 0.01^{(a)}$	$49.28 \pm 2.48^{(a,b)}$
(50 μM)	$2.73 \pm 0.01^{(a,b)}$	$3.58 \pm 0.01^{(a,b)}$	$60.12 \pm 1.20^{(a,b)}$

Data are expressed as means ± SE obtained in four experiments.
[a] Denotes statistical differences versus control and [b] *vs.* lipid peroxidation.

Table 2. IC_{50} tamoxifen concentrations required to inhibit lipid peroxidation in the absence or presence of melatonin (1 mM)

	Tamoxifen (μM)	Tamoxifen + Melatonin (1 mM)
1/Polarization	169.7	117.1
1/Anisotropy	171.0	119.7
MDA+4-HDA	111.2	51.5

Values are obtained from Figs. 1–3.

Discussion

Under the conditions of these studies, peroxidative stress in hepatic microsomal membranes promoted a decrease in membrane fluidity. These results are consistent with other reports in which various models of lipid peroxidation were shown to induce rigidity as measured by fluorescence polarization [24, 30, 38, 57] and electron spin resonance spectroscopy techniques [7, 17, 66]. Alterations in the chemical structure of membranes due to the formation of covalent cross-linkings between adjacent lipids and a reduction in the unsaturation/saturation ratio of fatty acids are considered possible causes of the decrease in membrane fluidity [7, 17, 24]. Hence, antioxidant molecules that inhibit lipid peroxidation also prevent membrane rigidity induced by oxidative stress. Melatonin [29], tocopherol [28, 38] and the enzymes superoxide dismutase and catalase [66] are several examples of antioxidants that make membranes more resistant to peroxidative damage and thus stabilize them. From the present results it is evident that tamoxifen, melatonin and a combination of the two prevent microsomal membrane rigidity and, in a parallel manner, they inhibit lipid peroxidation.

Microsomal lipid peroxidation is a complex process wherein hydroxyl radicals ($\cdot$OH), peroxyl radicals (LOO$\cdot$) and the perferryl ion have been implicated [37, 42, 71]. Recent studies have shown that melatonin efficiently scavenges both $\cdot$OH [61] and perhaps also LOO$\cdot$ [49] as well as other free radicals [43] through an electron donation reaction [50]. Moreover, the indolyl-like radical generated during the scavenging process [58] perhaps is also able to detoxify superoxide radicals thereby limiting the possibility of $\cdot$OH formation by the Fenton reaction [33]. On the other hand, tamoxifen inhibits microsomal lipid peroxidation [68, 69] presumably through mechanisms similar to those of melatonin by scavenging $\cdot$OH and LOO$\cdot$ by electron transfer [19]. Common modes of action of melatonin and tamoxifen in inhibiting lipid peroxidation might explain the enhanced response to tamoxifen in preventing membrane rigidity when melatonin is also available.

Besides the cooperative action of tamoxifen and melatonin in stabilizing membranes, Kumar et al. [39]

crease in membrane fluidity and MDA+4-HDA formation by 50%, i.e., IC_{50}s, are presented in Table 2.

The effects of the addition of 1 mM of melatonin with tamoxifen to the incubation medium are summarized in Table 3. Additionally, this table illustrates the ability of melatonin (1 mM) to resist microsomal membrane rigidity and MDA+4-HDA formation; for both parameters melatonin's efficiency was about 30%. As indicated in Table 3, the combination of tamoxifen and melatonin was highly effective in reducing membrane rigidity and lipid peroxidation. Thus, melatonin (1 mM) in combination with tamoxifen (150, 175 or 200 μM) totally prevented the changes in membrane fluidity (both $1/P$ and $1/A$) and lipid peroxidation induced by the free radical generating agents. This contrasts with the data in Table 1 which indicates that while the concentrations of tamoxifen used reduced the changes in membrane fluidity and lipid peroxidation, when melatonin and tamoxifen were used simultaneously a greater inhibition of the induced changes was apparent (Table 3).

Figures 1–3 illustrate the efficiency of tamoxifen (50–200 μM) with or without the addition of melatonin (1 mM) to inhibit decreases in membrane fluidity and increases in MDA+4-HDA formation. When melatonin was added, at each concentration of tamoxifen its stabilizing effects on the cell membrane (as indicated by less rigid membranes) and resistance to lipid peroxidation (as indicated by lower MDA+4-HDA levels) were significantly increased. Thus, the IC_{50} for tamoxifen decreased when melatonin was added (Table 2). No differences in membrane fluidity or MDA+4-HDA concentrations were observed between control microsomes for the two experiments; also, the level of lipid peroxidation did not differ between the control samples.

Table 3. Effect of the addition of melatonin on the ability of tamoxifen to prevent microsomal rigidity and to inhibit MDA+4-HDA formation

	1/P	1/A	MDA+4-HDA (nmol/mg protein)
Control	3.22 ± 0.00	4.33 ± 0.01	3.83 ± 0.49
Induced lipid peroxidation	$2.77 \pm 0.02^{(a)}$	$3.65 \pm 0.02^{(a)}$	$85.30 \pm 2.44^{(a)}$
Induced lipid peroxidation + melatonin (1 mM)	$2.91 \pm 0.00^{(a,b)}$	$3.86 \pm 0.02^{(a,b)}$	$57.65 \pm 3.64^{(a,b)}$
Induced lipid peroxidation + melatonin (1 mM) + tamoxifen			
(200 μM)	$3.22 \pm 0.02^{(b)}$	$4.34 \pm 0.02^{(b)}$	$3.99 \pm 0.35^{(b)}$
(175 μM)	$3.22 \pm 0.01^{(b)}$	$4.31 \pm 0.01^{(b)}$	$4.17 \pm 0.36^{(b)}$
(150 μM)	$3.12 \pm 0.05^{(b)}$	$4.17 \pm 0.08^{(b)}$	$11.64 \pm 5.00^{(b)}$
(100 μM)	$2.93 \pm 0.01^{(a,b)}$	$3.87 \pm 0.03^{(a,b)}$	$32.05 \pm 1.22^{(a,b)}$
(50 μM)	$2.88 \pm 0.02^{(a,b)}$	$3.83 \pm 0.02^{(a,b)}$	$45.50 \pm 1.05^{(a,b)}$

Data are expressed as means ± SE obtained in four experiments.
[a] Denotes statistical differences *vs.* control and [b] *vs.* lipid peroxidation.
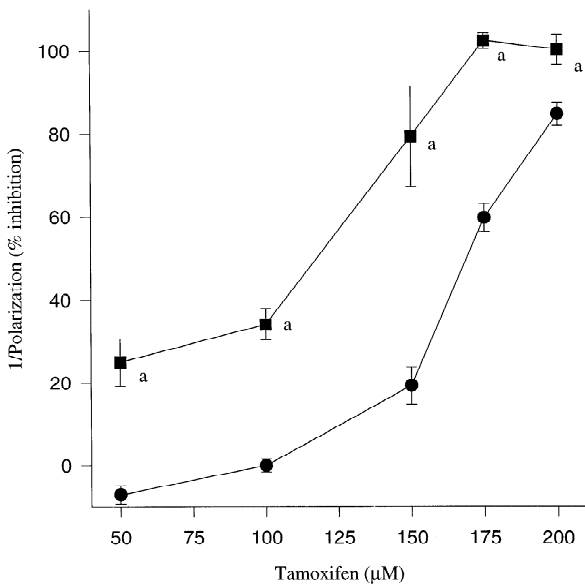

Fig. 1. Ability of tamoxifen (●) and tamoxifen plus melatonin (■) to prevent the decreases in microsomal membrane fluidity (1/P) induced by free radical generating agents. Percentage inhibitions are expressed as means ± SE obtained in four independent experiments. [a] Denotes statistical differences ($P < 0.05$).

Fig. 2. Inhibition of membrane rigidity (1/A) by tamoxifen in absence (●) or presence (■) of melatonin. Percentage inhibitions are expressed as means ± SE obtained in four experiments. [a] Denotes statistical differences ($P < 0.05$).

reported significant decreases in antioxidative enzymatic systems, i.e., glutathione peroxidase, catalase and superoxide dismutase, in postmenopausal women with breast cancer. Melatonin, whose synthesis decreases with age, is also known for its modulatory effect on enzymes involved in the free radical metabolism, either stimulating defensive enzymes such as glutathione peroxidase [3] and superoxide dismutase [2] or inhibiting those related to free radical promotion, e.g., nitric oxide synthase [4, 51]. Similarly, an increase in enzyme activities protective against free radicals has been reported during tamoxifen treatment [63].
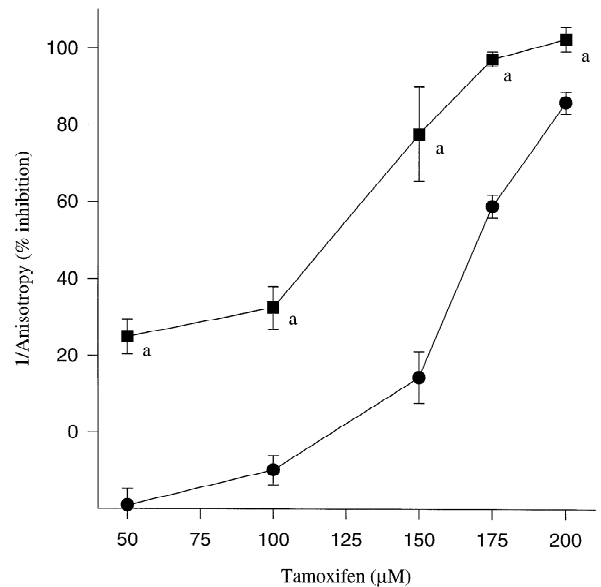
In the previous study [29] where we tested the role of melatonin in preserving hepatic microsomal membrane fluidity induced by free radicals and, utilizing the same methodological procedures reported here, melatonin (1 mM) was equally efficient as in the present study. Additionally, when hepatic microsomes were incubated with melatonin in the absence of agents which induce oxidative stress, even at high concentrations melatonin did not alter membrane fluidity significantly. The results obtained in the present study are consistent with these observations since the addition of melatonin to tamoxifen (150–200 μM) returned membrane fluidity to the

basal level, presumably the optimal level, in close relation with the inhibition of MDA+4-HDA formation (Figs. 1–3). The importance of maintaining a basal level of lipid peroxidation and therefore optimal membrane fluidity resides in the close relation of numerous membrane functions with membrane fluidity. Even slight changes in membrane fluidity may cause aberrant function and pathological processes [13, 21, 36, 64]. In contrast to the effects of melatonin, some scavenging molecules have been reported to modify the membrane fluidity even when basal levels of lipid peroxidation exist [44, 47, 48]. Tamoxifen also has been reported to decrease membrane fluidity in liposomes [18, 70], presumably by duplicating the effect of cholesterol, a major membrane component which induces rigidity when its intramembraneous concentrations rise [70]. Perhaps this property of tamoxifen may explain, in addition to possible methodological differences, the unexpectedly high IC_{50} concentration when calculated from the membrane fluidity parameters than would be predicted from the levels of MDA+4-HDA (Table 3). Clark et al. [9] reported a decrease in membrane fluidity when tamoxifen was added to culture medium containing breast cancer cells characterized by their invasive ability. Since the relationship of membrane fluidity to the metastatic potential of cancer cells is unclear [59], further research is needed to clarify this association.

While tamoxifen's clinical benefits and its relatively high tolerance level justifies its clinical use, several investigations have demonstrated that tamoxifen may also function as a genotoxic carcinogen [32, 67]. Thus, epidemiological studies have reported a rise in the incidence of proliferative and neoplastics lesions in the endometrium during treatment with tamoxifen [20, 26]. Because of this, there is interest in molecules that may allow the use of lower doses of tamoxifen thereby possibly reducing its side effects. The antioxidant ability of melatonin as well as the pharmacological advantages it seems to afford, i.e., its very low toxicity and the ease with which it can be administered, suggest its potential utility in therapy for free radical-related diseases [55, 56]. Since melatonin seems to concentrate in the nucleus of cells [45] its possible use in preventing the initial damage to DNA by free radicals that may lead to cancer also seems feasible. Indeed, Tan et al. [62] demonstrated that melatonin reduces DNA adduct formation induced by the carcinogen safrole while Vijayalaxmi et al. [65] showed that melatonin reduces chromosomal aberrations in gamma-irradiated human lymphocytes.

The present findings show a close functional association between tamoxifen and melatonin. It seems clear that tamoxifen's efficacy in decreasing microsomal membrane fluidity induced by lipid peroxidation is enhanced in the presence of melatonin. The ability of these molecules to cooperatively stabilize membrane fluidity
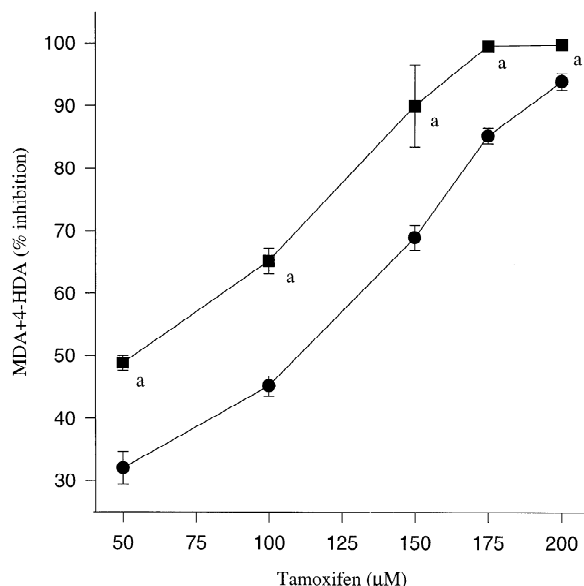

Fig. 3. Dose-dependent response to tamoxifen (●) or tamoxifen and melatonin (■) in reducing lipid peroxidation (expressed as MDA+4-HDA concentration). Percentage inhibitions are expressed as means ± SE obtained in four experiments. [a] Denotes statistical differences ($P < 0.05$).

would protect the membranes from free radical damage and preserve membrane functions which are modulated by membrane fluidity. These data add new information related to the known cooperative actions of tamoxifen and melatonin.

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