Role of Pinoline and Melatonin in Stabilizing Hepatic Microsomal Membranes against Oxidative Stress[1]

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We investigated the influence of pinoline (0.01–1.5 mM) on microsomal membrane fluidity before and after rigidity was induced by oxidative stress. In addition, we tested the effect of pinoline in the presence of 1 mM melatonin. The fluidity in rat hepatic microsomes was monitored using fluorescence spectroscopy and it was compared to the inhibition of malonaldehyde (MDA) plus 4-hydroxyalkenals (4-HDA) production as a reflection of lipid peroxidation. Below 0.6 mM, pinoline inhibited membrane rigidity in a manner parallel to its inhibitory effect on MDA + 4−HDA formation. At concentrations between 1–1.5 mM, pinoline was less effective in stabilizing microsomal membranes than was predicted from its inhibition of lipid peroxidation. The addition of 1 mM melatonin enhanced the membrane-stabilizing activity of pinoline (0.01–0.6 mM). This cooperative effect was not observed for concentrations of pinoline between 1–1.5 mM. When pinoline was tested without induced oxidative damage, 1–1.5 mM pinoline maintained membrane fluidity at the same level as that recorded after induced lipid peroxidation. The results suggest that pinoline may be another pineal molecule that prevents membrane rigidity mediated by lipid peroxidation and this ability is enhanced by melatonin.

INTRODUCTION

Numerous free radicals are generated during mitochondrial respiration and endoplasmic reticulum metabolism (Yu, 1994). Lipid peroxidation is the expression of free radical damage in cell membranes. The biochemical reaction is a degenerative process in which the acyl chains of the phospholipids are especially vulnerable to free radical attack. As a result of lipid peroxidation, several products including peroxyl radicals (LOO•), endoperoxides, and hydroperoxides are generated. Some of these products are sufficiently toxic to propagate the oxidative process using adjacent phospholipids as substrates (Curtis *et al.*, 1984; Gutteridge, 1995).

Structural changes in cell membranes produced during lipid peroxidation disrupt molecular motion in the membrane (Yu *et al.*, 1992). Oxidative stress in microsomes increases order in the bilayer, a parameter that reflects the mean angular deviation from the bilayer plane of the fatty acid chain, which is inversely correlated with membrane fluidity (Curtis *et al.*, 1984).

Given that the dynamics of the lipid bilayer modulates numerous cell functions, e.g., activity of enzymes associated with the membrane, solute transport, and signal transduction, it is important for cell viability to

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maintain the membranes at optimal fluidity (Cooper, 1977; Van Blitterswijk, 1985). Thus, there is considerable interest in antioxidative molecules, which are able to stabilize membranes because of their protective role against lipid peroxidation.

N-Acetyl-5-methoxytryptamine (melatonin) (Fig. 1A) is the main secretory product of the mammalian pineal gland (Reiter, 1991). Melatonin has been classically implicated in the control of biological rhythms and in the modulation of several neuroendocrine and immunological functions (Reiter, 1991; Maestroni, 1993). Over the last 6 years, the antioxidant ability of melatonin has been well documented (for review see Reiter, 1995, 1998). Melatonin is a widely acting scavenger molecule, which is able to detoxify several reactive oxygen species (Tan *et al.*, 1993; Poeggeler *et al.*, 1994; Matuszak *et al.*, 1997; Cuzzocrea *et al.*, 1998). Moreover, melatonin exhibits antioxidant activity because it stimulates several enzymes related to the antioxidant defense system (Antolin *et al.*, 1996; Reiter *et al.*, 1997) while inhibiting other enzymes implicated in the free radical generation (Bettahi *et al.*, 1998).

Pinoline (Fig. 1B) is structurally related to melatonin. Pinoline formation has been proposed via Pictet–Spengler reaction by condensation between indoleamines and aldehydes (Hardeland *et al.*, 1993; Callaway *et al.*, 1994; Pähkla *et al.*, 1996). Several laboratories claim that pinoline is present in the pineal gland and in other tissues as well (Shoemaker *et al.*, 1978; Kari, 1981; Langer *et al.*, 1984). Pinoline has been shown to increase brain serotonin levels because it inhibits monoamine oxidase; it also reduces uptake of serotonin in the central nervous system (Airaksinen *et al.*, 1978; Langer *et al.*, 1984). Recently, antioxidant properties of several β-carbolines and pinoline have been proposed (Kawashima *et al.*, 1995; Pähkla *et al.*, 1998; Pless *et al.*, 1999).

In the present paper we report, using microsomes isolated from rat liver, the effect of pinoline alone or in combination with melatonin on membrane fluidity associated with lipid peroxidation. Moreover, we tested the effect of pinoline on membrane fluidity in the absence of lipid peroxidation. Fluorescence polarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfonate (TMA-DPH) was used to estimate the changes in the microsomal membrane fluidity.

METHODS

Chemicals and Solutions

Pinoline, $FeCl_3$, ADP, NADPH, and EDTA were obtained 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 purchased from Caymen Chemical (Ann Arbor, MI). Other chemicals utilized were of the highest analytical grade and were purchased from commercial sources. Pinoline and melatonin were diluted in methanol and TMA-DPH in tetrahydrofuran and water. Methanol and tetrahydrofuran were 2 and 0.4% in the final mixture, respectively. $FeCl_3$, ADP, NADPH, and EDTA were diluted in the incubation buffer. Pinoline, melatonin, $FeCl_3$, ADP, and NADPH solutions were prepared fresh just before use.

Animals and Isolation of Microsomes

Hepatic microsomal membranes were obtained from male Sprague-Dawley rats weighing 225–250 g. Animals were purchased from Harlan and received
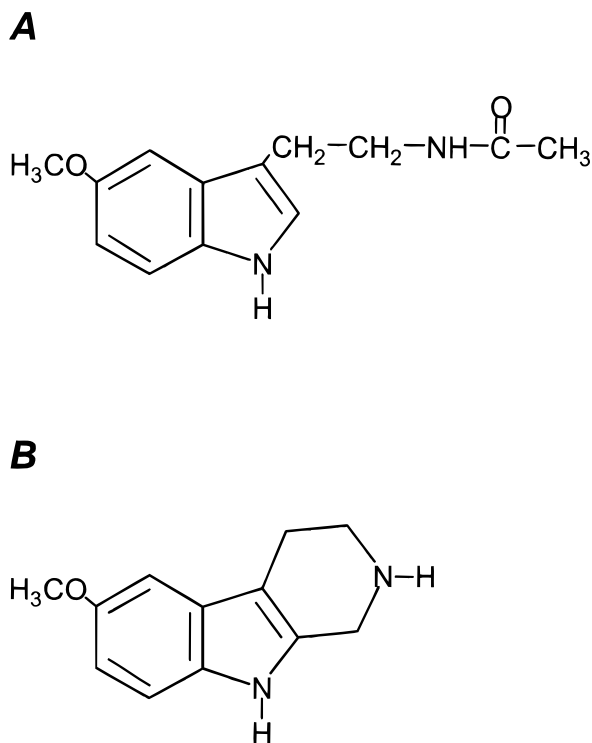
A

B

Fig. 1. Structure of melatonin (A) and its tricyclic metabolite pinoline (B).

standard food and water *ad libitum*. After being acclimated for 1 week, the animals were sacrificed by decapitation, a procedure approved by the U. S. Departments of Agriculture, and Health and Human Service and the Institutional Animal Care and Use Committee. The liver was quickly removed, frozen in liquid nitrogen, and stored at $-80°C$.

The microsomal fraction was isolated as described previously (García *et al.*, 1997). Briefly, the liver was homogenized 1/10 w/v in 140 mM KCl/ 20 mM HEPES buffer (pH 7.4). The suspension was centrifuged at $1000 \times g$ for 10 min and the resulting supernatant was centrifuged at $105,000 \times g$ for 60 min. The pellet obtained was resuspended in the buffer and centrifuged at $10,000 \times g$ for 15 min. Then the supernatant was recentrifuged at $105,000 \times g$ for 60 min and the final pellet was resuspended 1/1 v/v and stored at $-80°C$ until assay. After isolation, microsomal membrane fluidity and MDA + 4 − HDA concentrations were measured with and without a free radical-generating system.

Peroxidation of the Microsomal Membranes

In the first study ($n = 4$), microsomal membranes (0.5 mg/ml) were suspended in 50 mM Tris-HCl buffer (pH 7.4). After incubation in a water bath for 30 min at 37°C with pinoline (0.01, 0.1, 0.3, 0.6, 1, and 1.5 mM), lipid peroxidation was induced by addition of $FeCl_3$ (0.2 mM), ADP (1.7 mM) and NADPH (0.2 mM) followed by incubation for 20 min at 37°C under aerobic conditions. Lipid peroxidation was stopped by addition of EDTA (2 mM). Control microsomes with and without induced lipid peroxidation were exposed under the same incubation conditions as those treated with pinoline.

In other experiments ($n = 4$), melatonin (1 mM) was added at the same time as was pinoline. In this study, melatonin was also tested in the absence of pinoline. Identical procedures were conducted as in the previous experiments, i.e., pinoline, $FeCl_3$, ADP, and NADPH concentrations and incubations conditions.

Finally, the effects of pinoline at a uniform concentration, were examined in the absence of induced lipid peroxidation ($n = 4$).

Measurements of Fluidity and MDA + 4 − HDA Concentrations in the Microsomal Membranes

Microsomal fluidity was monitored by fluorescence spectroscopy. Labeling of the membranes was performed according to the method of Yu *et al.* (1992) as follows. A suspension of 0.5 mg microsomal protein in 50 mM Tris-HCl buffer (pH 7.4) (3 ml) was vigorously mixed with TMA-DPH (66.7 nM) for 1 min and incubated with shaking at 37°C for 30 min to ensure the uniform distribution of the fluorescent probe in the microsomes. Polarization parameters (average of 30 observations for each determination) were carried out in a Perkin–Elmer LS-50 Luminiscence Spectrometer. TMA-DPH was excited at 360 nm and its emission recorded at 430 nm. The cuvette temperature was maintained at $2 \pm 0.01°C$ during the assay using a circulator bath. The degree of polarization (P) was calculated using the equation:

$$ P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}} $$

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 a correction factor for the optical system. Results of membrane fluidity were expressed as the inverse of P (Yu *et al.*, 1992).

Measurements of MDA + 4 − HDA concentrations are an index of lipid peroxidation in biological membranes (Esterbauer and Cheeseman, 1990). These products were determined by the colorimetric assay mentioned above. MDA and 4-HDA react with a chromogenic reagent at 45°C yielding a stable chromophore with maximal absorbance at 586 nm wavelength. Results are expressed as MDA + 4 − HDA nmol mg^{-1} microsomal protein. Protein concentrations were measured by the Bradford (1976) method using bovine serum albumin as standard.

Statistics

Values are shown as means $\pm$ standard error (SE). Student's *t*-tests, for paired or unpaired as appropriate, were used for comparison of the means. The level of significance was defined as $p < 0.05$.

RESULTS

Effects of Pinoline on the Membrane Fluidity After Induced Lipid Peroxidation

After incubation of the microsomal membranes with $FeCl_3$, ADP, and NADPH, membrane fluidity

decreased and lipid peroxidation was induced, as indicated by elevation of MDA + 4 − HDA concentrations, when compared to those in microsomes treated in absence of the oxidizing reagents.

The ability of pinoline in preventing membrane rigidity and the lipid peroxidation are illustrated as percentage inhibition in Fig. 2. Preincubation of the membranes with pinoline (0.01–1.5 mM) prior to induction of the lipid peroxidation reduced microsomal rigidity although the activity of 1 and 1.5 mM pinoline showed a drop in its stabilizing ability. Pinoline (1.5 mM) was even less active than 0.3–1 mM pinoline (Table I).

In contrast to the effect on membrane fluidity, progressively increasing concentrations of pinoline prevented MDA + 4 − HDA formation in a concentration-dependent manner. The concentration of β-carboline required to inhibit the formation of MDA + 4 − HDA by a 50%, i.e., IC$_{50}$, was 0.391 mM. Complete inhibition of lipid peroxidation was achieved when the concentrations of pinoline were above 1 mM.
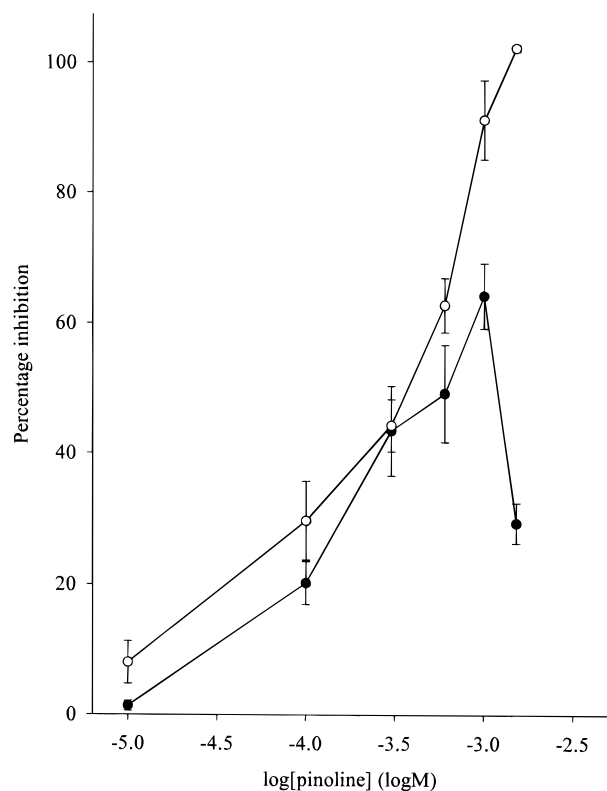

Fig. 2. Ability of pinoline to prevent the decrease in microsomal membrane fluidity (●) and MDA + 4 − HDA formation (○) after lipid peroxidation induction. Percentage are expressed as means ± SE obtained in four independent experiments.

Effects of Pinoline in the Presence of 1 mM Melatonin in Stabilizing Microsomal Membranes Against Lipid Peroxidation

Table II summarizes the effects of the addition of 1 mM melatonin simultaneously with pinoline on the microsomal membrane fluidity and MDA + 4 − HDA concentrations. Moreover, this table shows the ability of 1 mM melatonin to reduce membrane rigidity in the absence of pinoline. Melatonin was used alone, for both rigidity and MDA + 4 − HDA formation; the protective activity of melatonin was about 30%.

The administration of melatonin in combination with pinoline prior to the induction of lipid peroxidation enhanced the response of 0.001–0.6 mM pinoline in stabilizing microsomal membranes. On the contrary, melatonin did not improve the activity of pinoline in reducing the rigidity of the microsomal membranes when pinoline concentrations were 1 and 1.5 mM (Fig. 3). Pinoline (1 and 1.5 mM) plus 1 mM melatonin preserved the fluidity at about 69 and 33%, respectively; these results are similar to those obtained when the β-carboline was used alone (Figs. 2 and 3).

These data contrast with the ability of the compounds to prevent MDA + 4 − HDA formation. Melatonin enhanced the response of each concentration of pinoline in resisting lipid peroxidation until maximal protection was achieved. Under these conditions, the half-maximal inhibition value of pinoline in the presence of 1 mM melatonin was reduced to 0.141 mM.

There were no statistically significant differences in the fluidity and MDA + 4 − HDA contents in control microsomes in the absence and presence of lipid peroxidation for these experiments.

Effects of Pinoline on Microsomal Membrane Fluidity and MDA + 4 − HDA Concentrations

Figure 4A illustrates the effect of pinoline on membrane fluidity. The polarization parameter was minimally affected by pinoline at concentrations less than 0.6 mM, implying that pinoline did not influence membrane fluidity below this concentration. However, when pinoline concentrations added to incubation buffer were increased above 0.6 mM, membrane fluidity decreased significantly.

Determinations of MDA + 4 − HDA values in the absence of oxidative stress in microsomes incubated with 0.01–1.5 mM pinoline yielded values even
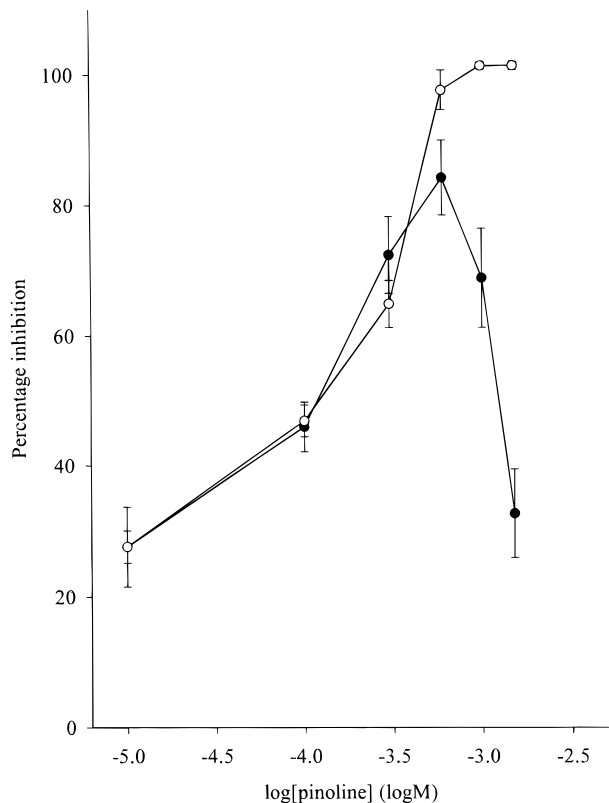
Table I. Effect of Pinoline on the Membrane Fluidity and MDA + 4 − HDA Concentration in Hepatic Microsomes Treated with Agents to Induce Lipid Peroxidation

	Membrane fludity (polarization^{-1})^a	MDA + 4 − HDA (nmol mg^{-1}Pr)^a
Control	3.030 ± 0.017	3.575 ± 0.259
Induced lipid peroxidation	2.695 ± 0.013(*)	111.902 ± 10.555(*)
Induced lipid peroxidation + pinoline 1.5 mM	2.803 ± 0.017$^{(*,\dagger)}$	1.290 ± 0.690$^{(*,\dagger)}$
1 mM	2.908 ± 0.007$^{(*,\dagger)}$	12.615 ± 6.615$^{(\dagger)}$
0.6 mM	2.857 ± 0.018$^{(*,\dagger)}$	43.990 ± 5.982$^{(*,\dagger)}$
0.3 mM	2.838 ± 0.019$^{(*,\dagger)}$	62.952 ± 7.201$^{(*,\dagger)}$
0.1 mM	2.763 ± 0.005$^{(*,\dagger)}$	80.925 ± 12.681$^{(*,\dagger)}$
0.01 mM	2.700 ± 0.022$^{(*,\dagger)}$	103.780 ± 11.868(*)

^{a} (*) Denotes statistical differences ($p < 0.05$) versus control and (†) versus lipid peroxidation; $n = 4$ experiments.

less that those incubated in the absence of pinoline (Fig. 4B).

DISCUSSION

The principle of the fluorescence spectroscopy studies relies on the intercalation into the membrane of a fluorescent molecule which, when illuminated by polarized light, emits a fluorescence signal. The degree of polarization depends of the state of mobility on the probe, which reflects motion in the membrane lipid environment (Zimmer *et al.* 1993).

Loss of freedom of motion in membranes after oxidative stress is well documented using biological and liposomal membranes (Dobretsov *et al.*, 1977; Curtis *et al.*, 1984; Chen and Yu, 1994). Two structural reasons have been advanced as causal relationships of the loss of membrane fluidity during oxidative stress.

First, there may be a drop in the polyunsaturated/saturated fatty acid ratio in membrane composition (Curtis *et al.*, 1984) as a consequence of the higher susceptibility of the polyunsaturated fatty acids to the oxidative stress (Gutteridge, 1995); second, the formation of cross linking among the membrane lipid moieties may limit motion (Chen and Yu, 1994). In the present study, when we incubated hepatic microsomes with FeCl$_3$/NADPH/ADP, membrane fluidity decreased.

Pinoline and melatonin, which are produced in the pineal gland and possibly other organs as well, assist microsomes in resisting membrane rigidity induced by lipid peroxidation. This paper provides the first evidence that pinoline stabilizes biological membranes. The role of melatonin in preserving membrane fluidity against oxidative damage was tested previously in our laboratory. Using the same microsomal and free radicals-generating model, 1 mM mela-

Table II. Effect of the Addition of Melatonin on the Ability of Pinoline in Preventing the Microsomal Rigidity and Inhibiting MDA + 4 − HDA Formation in Hepatic Microsomal Membranes

	Membrane fluidity (polarization^{-1})^a	MDA + 4 − HDA (nmol mg^{-1} protein)^a
Control	2.997 ± 0. 009	2.622 ± 1.183
Induced lipid peroxidation	2.685 ± 0.014$^{(*)}$	99.570 ± 6.117$^{(*)}$
Induced lipid peroxidation + melatonin 1mM	2.773 ± 0.012$^{(*,\dagger)}$	75.552 ± 5.992$^{(*,\dagger)}$
Induced lipid peroxidation + melatonin 1mM + pinoline 1.5 mM	2.709 ± 0.013$^{(*,\dagger)}$	1.400 ± 0.967$^{(\dagger)}$
1 mM	2.908 ± 0.013$^{(*,\dagger)}$	1.403 ± 0.895$^{(\dagger)}$
0.6 mM	2.947 ± 0.011$^{(\dagger)}$	5.232 ± 3.909$^{(\dagger)}$
0.3 mM	2.910 ± 0.016$^{(*,\dagger)}$	37.280 ± 5.853$^{(*,\dagger)}$
0.1 mM	2.830 ± 0.010$^{(*,\dagger)}$	54.257 ± 4.407$^{(*,\dagger)}$
0.01 mM	2.778 ± 0.014$^{(*,\dagger)}$	76.853 ± 7.005$^{(*,\dagger)}$

^{a} (*) Denotes statistical differences ($p < 0.05$) versus control and (†) versus lipid peroxidation; $n = 4$ experiments.

Fig. 3. Response of pinoline, in addition to 1 m*M* melatonin, in preserving the membrane fluidity (●) and inhibiting the MDA + 4 − HDA (○) after oxidative stress. Results are expressed as percentage mean ± SE obtained in four experiments.

Fig. 4. Effects of pinoline on microsomal membrane fluidity (A) and MDA + 4 − HDA concentrations (B).

tonin prevented membrane rigidity with the same efficiency as in this study, about 30% (García *et al.*, 1997). Identical results were obtained when we tested the cooperative action of melatonin and tamoxifen in stabilizing microsomal membranes; tamoxifen is an antiestrogenic drug used in breast cancer therapy (García *et al.*, 1998).

Since pinoline (0.01–0.6 m*M*) prevented MDA + 4 − HDA formation equally effectively as it prevented membrane rigidity, it seems reasonable to suggest that, at these pinoline concentrations, its ability to scavenge free radicals may be a mechanism by which it increases membrane fluidity. Some other molecules, e.g., melatonin, tocopherol, stobadine, catalase, and superoxide dismutase, have also been shown to stabilize membranes against lipid peroxidation because of their antioxidant properties (Watanabe *et al.*, 1990; Zimmer *et al.*, 1993; Kaplán *et al.*, 1993; García *et al.*, 1997).

Lipid peroxidation in microsomes is an autooxidative chain reaction, wherein hydroxyl radicals (·OH) and LOO· are involved in a NADPH-dependent pro-

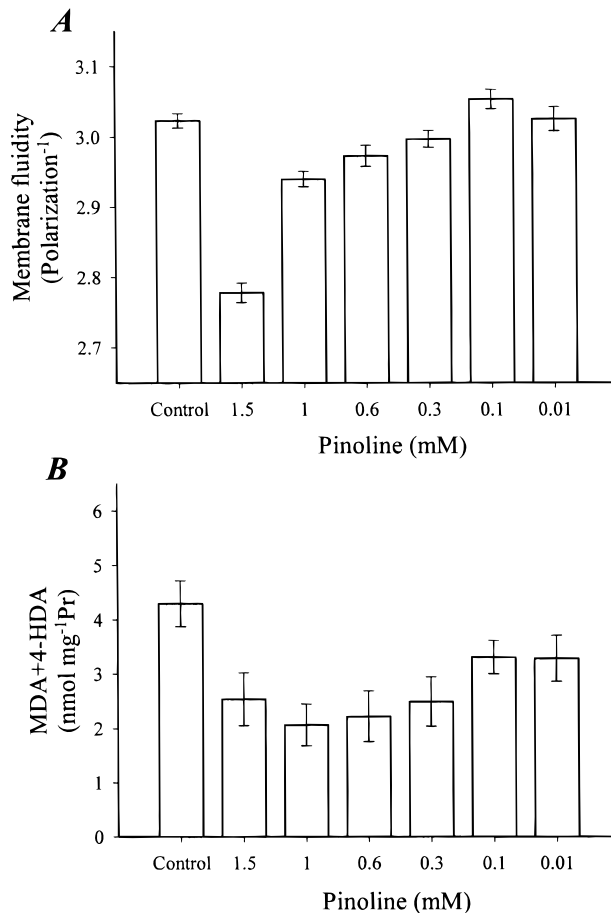cess (Yu, 1994). Both, melatonin and pinoline had been shown to detoxify ·OH and LOO·. Tan *et al.* (1993, 1998) and others (Matuszak *et al.*, 1997; Stasica *et al.*, 1998) found that melatonin is a powerful scavenger of ·OH. In addition, melatonin prevented the peroxidation of liposomes treated with $FeCl_3$ and ascorbic acid (Marshall *et al.*, 1996). One mechanism proposed to explain the antioxidant ability of melatonin may be electron donation, since melatonin is rapidly oxidized in the presence of free radicals (Poeggeler *et al.*, 1994). Previous studies also claim that pinoline, *in vitro*, limits lipid peroxidation. Pinoline is a powerful molecule in reducing lipid peroxidation in brain homogenates incubated with H_2O_2, in one study being even more active than melatonin (Pless *et al.*, 1999). By contrast, pinoline seems to be less potent that melatonin in scavenging ·OH; this was studied in a system devoid of membranes, which used terephthalic acid as a dosimeter of ·OH (Pähkla *et al.*, 1998). The different

efficiencies in protecting against oxidative damage must be related to the chemical ability for accepting or donating electrons unique for each of these molecules and to their relative water/lipid solubility coefficients. Other indoles synthesized from the essential amino acid tryptophan in the mammalian pineal gland also have been shown to scavenge free radicals, although with less efficiency than melatonin, e.g., 5-hydroxytryptamine (Marshall *et al.*, 1996), *N*-acetyl-5-hydroxytryptamine, and 5-methoxytryptamine (Tan *et al.*, 1993). Thus it is possible that the antioxidant role of the pineal gland may be a result of the cooperative antioxidant properties of several of its metabolic products.

The highest pinoline concentrations (1 and 1.5 mM) failed to stabilize microsomal membranes despite their inhibition of lipid peroxidation as indicated by reduced MDA $+ 4 -$ HDA formation (Fig. 2). These results differ from our previous observations obtained with melatonin, which stabilized membranes at the optimal level as reflected by microsomal fluidity in the absence of oxidative stress (García *et al.*, 1997). The lack of a response of 1 and 1.5 mM pinoline in preserving membrane fluidity but efficiently protecting against lipid peroxidation may be a consequence of the direct interaction of pinoline with the microsomal lipid bilayer. Indeed, when experiments were carried out in presence of 1 mM melatonin with the aim of increasing antioxidant protection in the incubation medium, fluidity levels were obtained that were similar to those reported for pinoline alone. To assess this presumption, we incubated microsomes with pinoline (0.01–1.5 mM) in the absence of oxidative reagents. Similar results were obtained for 1 and 1.5 mM pinoline as those recorded after lipid peroxidation had been induced.

Tocopherols, like pinoline, are able to perturb molecular motion in the membrane. Ohyashiki *et al.* (1986) showed that the addition of α-tocopherol to membranes isolated from porcine intestinal brush border may reduce the mobility of the pyrene molecules because of the interaction of α-tocopherol with the membrane lipids. Some vitamin E derivatives are even more effective than cholesterol in disrupting the membrane (Massey *et al.*, 1982; Ohki *et al.*, 1984). A similar behavior was detected using tamoxifen and also its more active metabolite 4-hydroxytamoxifen; both these compounds are believed to decrease fluidity by an effect that mimics that of cholesterol (Wiseman *et al.*, 1993).

In contrast with the alteration in fluidity caused by pinoline, tocopherols, and tamoxifen, we have previously reported that melatonin did not change the polarization parameter and, therefore, fluidity levels in microsomes in basal conditions of oxidative stress (Garcia *et al.*, 1997). Using electron spin resonance methods, melatonin also did not modify the signal of spin labels placed at the 5th and 12th carbon of phospholipids from synaptosomal membranes indicating that melatonin does not disturb the degree of membrane organization at those levels (Costa *et al.*, 1997). The importance of preservation of optimal levels of membrane fluidity resides in the close correlation between fluidity and membrane function as indicated above. Even small deviations in membrane fluidity are associated with loss of important functions (Van Blitterswijk, 1985).

Besides melatonin's role in stabilizing membranes against lipid peroxidation, melatonin has other advantages because of its ubiquitous distribution in every cellular compartment (Menéndez-Peláez and Reiter, 1993) which is a result of the ease with which it crosses lipid bilayers (Costa *et al.*, 1995). This suggests the possibility that melatonin may stabilize all cell membranes.

In conclusion, the data reported here provides evidence that pinoline (below 0.6 mM) *in vitro* is able to protect against changes in membrane fluidity in hepatic microsomes associated with oxidative damage. At high concentrations, above 0.6 mM, pinoline failed to stabilize microsomal membranes. The inability of pinoline at these concentrations to preserve fluidity, even when the antioxidant ability of pinoline was enhanced by melatonin, suggests that pinoline exerts a restrictive action on lipid dynamics that exceeds the inhibition of the rigidity that would be expected as a result of the antioxidant activity of pinoline. These results may become significant in understanding the role of pineal metabolites related to their protective effect in preventing oxidative damage even at physiological concentrations (Reiter, 1995).

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