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N-acetylserotonin suppresses hepatic microsomal membrane rigidity associated with lipid peroxidation

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Abstract

N-acetylserotonin, the immediate precursor of melatonin in the tryptophan metabolic pathway in the pineal gland, has been reported to be an antioxidant. The aim of this work was to test the effect of *N*-acetylserotonin in stabilizing biological membranes against oxidative stress. Hepatic microsomal membranes from male adult rats were incubated with *N*-acetylserotonin (0.001–3 mM) before inducing lipid peroxidation using $FeCl_3$, ADP and NADPH. Control experiments were done by incubating microsomal membranes with *N*-acetylserotonin in the absence of lipid peroxidation-inducing drugs. Membrane fluidity was assessed by fluorescence spectroscopy and malonaldehyde plus 4-hydroxyalkenals concentrations were measured to estimate the degree of lipid peroxidation. Free radicals induced by the combination of $FeCl_3$ + ADP + NADPH produced a significant decrease in the microsomal membrane fluidity, which was associated with an increase in the malonaldehyde plus 4-hydroxyalkenals levels. These changes were suppressed in a concentration-dependent manner when *N*-acetylserotonin was added in the incubation buffer. In the absence of lipid peroxidation, *N*-acetylserotonin (0.001–3 mM) did not change membrane fluidity nor malonaldehyde plus 4-hydroxyalkenals levels. These results suggest that the protective role of *N*-acetylserotonin in preserving optimal levels of fluidity of the biological membranes may be related to its ability to reduce lipid peroxidation.

Keywords: *N*-acetylserotonin; Lipid peroxidation; Membrane fluidity; Microsome

1. Introduction

Since the demonstration by Tan et al. (1993) of the antioxidant ability of melatonin, several reports claim that other pineal products may also exhibit antioxidant effects (Poeggeler et al., 1996; Pless et al., 1999; Qi et al., 2000). *N*-acetylserotonin is an indoleamine isolated from the pineal gland as well as from several extrapineal tissues such as the retina, digestive tract and ovary (Itoh et al., 1997). The concentration of *N*-acetylserotonin in mammalian plasma is in the nanomolar range (Younglai et al., 1986).

N-acetylserotonin is the immediate precursor of melatonin in the metabolism of tryptophan in the pineal gland. Chemically, *N*-acetylserotonin only differs from melatonin in the substitution of a hydroxy group for the methoxy group in position 5 of the indole ring (Fig. 1). Both indoleamines show light-controlled daily rhythms in their synthesis with peak production occurring at night and a nadir during the photophase. These rhythms are a consequence of the increased activity of serotonin *N*-acetyltransferase (E.C. 2.3.1.5) at night (Pang et al., 1984; Ho et al., 1985; Namboodiri et al., 1985; Míguez et al., 1996; Viswanathan et al., 1998). As occurs with melatonin, the pineal content of *N*-acetylserotonin declines with age (Pang et al., 1984; Míguez et al., 1998) and both indoleamines have protective effects against toxin-induced damage in several organs (Bachurin et al., 1999; López-González et al., 2000; Ohta et al., 2000; Montilla et al., 2000; Martín et al., 2000; Calvo et al., 2001).

The lipid dynamic of the biological membranes modulates essential cell functions including cell growth, solute transport, signal transduction and membrane-associated enzyme activities (Levi et al., 1990; Sunshine and McNamee, 1998; Boyan et al., 1999; Prasad et al., 1999; Emmerson et

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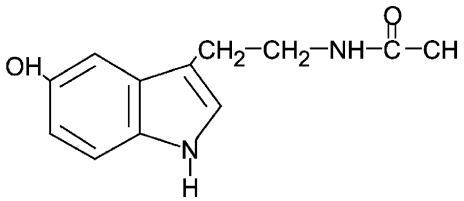

Fig. 1. Structure of *N*-acetylserotonin. This molecule has a hydroxy group in position 5 of the indole nucleus; by comparison, melatonin has a methoxy group in this position.

al., 1999; Baenziger et al., 2000; Parks et al., 2000; Oghalai et al., 2000). Structural changes in cell membranes produced by oxidative stress reduce membrane fluidity and influence pathological processes (Ghosh et al., 1993; Niranjan and Krishnakantha, 2000).

Aging is characterized by a decrease in physiological functions (Caprari et al., 1999). Although several hypothetical molecular mechanisms have been proposed to explain aging and aging-related diseases, it is widely accepted that free radicals and lipid peroxidation are involved in aging (Harman, 1984). Recently, it has been shown that during aging and in degenerative diseases, a disturbance of the phospholipid dynamic in the biological membranes occurs (Viani et al., 1991; Yu et al., 1992; Choe et al., 1995; Reiter et al., 1999; Lee et al., 1999; Hashimoto et al., 1999; Kanfer et al., 1999; Sennoune et al., 2000).

We have previously shown that melatonin, pinoline and 5-methoxytryptophol stabilize biological membranes against the injury caused by free radicals since these compounds preserve the lipid bilayer fluidity. Since little is known about the effect of *N*-acetylserotonin in the membrane, the purpose of this paper was to investigate the relationship of *N*-acetylserotonin to lipid motion in rat hepatic microsomal membranes obtained from rats and subjected to oxidative stress-induced lipid peroxidation.

2. Materials and methods

2.1. Materials

All chemicals were of the highest purity commercially available. *N*-acetylserotonin, $FeCl_3$, ADP, NADPH and EDTA were purchased from Sigma (St. Louis, MO), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene-sulfonate (TMA-DPH) was obtained from Molecular Probes (Eugene, OR). The lipid peroxidation assay kit was obtained from Calbiochem (San Diego, CA). *N*-acetylserotonin was dissolved in ethanol and TMA-DPH in tetrahydrofuran and water. Ethanol and tetrahydrofuran were 2% and 0.4% in the final mixture, respectively. The other drugs were diluted in the incubation buffer. Each solution was freshly prepared just prior to use.

2.2. Animals and microsomal fraction isolation

Male Sprague–Dawley rats weighing 220 ± 20 g were obtained from Harlan. Animals were housed according the guidelines outlined by NIH and all procedures used were approved by the Institutional Animal Care and Utilization Committee. Rat chow and water were available ad libitum. After acclimating for 2 weeks, the animals were killed by decapitation and the livers were immediately removed, frozen in liquid nitrogen and stored at -70 °C.

Microsomal fractions were isolated using the differential centrifugation method described by Yu et al. (1992). Livers were homogenized in 1/10 w/v in 140 mM KCl/20 mM HEPES buffer pH 7.4 and cleared of cell debris and nuclei by centrifugation at $1000 \times g$ for 10 min. The supernatant was centrifuged at $105{,}000 \times g$ for 60 min. The pellet containing the microsomal membranes and mitochondria was re-suspended in the same buffer and centrifuged at $10{,}000 \times g$ for 15 min to separate the mitochondrial fraction. The supernatant was centrifuged again at $105{,}000 \times g$ for 60 min and the resulting pellet, containing the microsomal membranes, was re-suspended 1/1 v/v and stored at -70 °C until its use.

2.3. Measurement of the membrane fluidity

Fluorescence spectroscopy studies have been extensively used in the assessment of the fluidity in biomembranes (Hashimoto et al., 1999). Membrane fluidity was determined by the fluorescence polarization technique previously described (Yu et al., 1992). Microsomal membranes were labeled with the probe TMA-DPH. This molecule includes a cationic trimethilamonium substituent in the DPH molecule and was designed to improve the localization of DPH in the membrane, since DPH is accumulated in the hydrophobic region of the phospholipid bilayer and TMA-DPH is intercalated in an orientation parallel to the long axis of the phospholipids with the cationic rest oriented to the surface. The probe, when illuminated by polarized light, emits a fluorescence signal, which reflects motion in the membrane lipid environment (Zimmer et al., 1993).

A total of 0.5 mg of microsomal protein sample suspended in 3 ml 50 mM Tris–HCl buffer pH 7.4 was mixed with 66.7 nM TMA-DPH and incubated at 37 °C for 30 min to uniformly distribute the probe into the microsomes. Fluorescence was monitored in a Perkin-Elmer LS-50B Luminiscence Spectrometer equipped with the polarization accessories. Samples were stirred constantly and the temperature maintained at 22 ± 0.1 °C. TMA-DPH was excited at 360 nm and the emission recorded at 430 nm. Polarization (average of 30 observations for each determination) was calculated using the following equation

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

Table 1
Effects of *N*-acetylserotonin (*N*-acetylserotonin) on microsomal membrane fluidity and malondialdehyde plus 4-hydroxyalkenals levels after the induction of lipid peroxidation

	Membrane fluidity (1/polarization) (nmol/mg protein)	Malondialdehyde plus 4-hydroxyalkenals
Control	3.145 ± 0.006	1.47 ± 0.43
Induced lipid peroxidation	2.962 ± 0.013[a]	51.55 ± 2.46[a]
Induced lipid peroxidation + *N*-acetylserotonin		
3 mM	3.160 ± 0.011[b]	3.53 ± 0.41[a,b]
1 mM	3.135 ± 0.006[b]	3.40 ± 0.38[a,b]
0.3 mM	3.047 ± 0.021[a,b]	18.55 ± 1.64[a,b]
0.1 mM	2.998 ± 0.022[a]	33.15 ± 2.49[a,b]
0.01 mM	2.960 ± 0.015[a]	47.00 ± 1.70[a]
0.001 mM	2.950 ± 0.015[a]	51.97 ± 1.51[a]

Values are the means ± S.E.M. of four experiments.
[a] $p < 0.05$ versus control.
[b] Versus lipid peroxidation.

where IV_V and IV_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. A statistically inverse correlation between polarization records and membrane fluidity has been proven and thus, changes in membrane fluidity are expressed as the reciprocal of polarization (Yu et al., 1992; Lee et al., 1999).

2.4. Malondialdehyde plus 4-hydroxyalkenals and protein determination

Malondialdehyde plus 4-hydroxyalkenals production was estimated as an index of oxidative damage. Their concentrations were determined in the microsomal suspension using a commercial kit. In this assay, malondialdehyde and 4-hydroxyalkenals react with *N*-methyl-2-phenylindole yielding a stable chromophore that absorbs light at 586 nm. Results are expressed as nmol malondialdehyde plus 4-hydroxyalkenals/mg microsomal protein. Proteins were assayed by the method of Bradford (1976) using bovine serum albumin as standard.

2.5. Induction of membrane lipoperoxidation

Microsomal membranes (0.5 mg/ml), suspended in 50 mM Tris–HCl buffered to pH 7.4, were incubated with *N*-acetylserotonin (0.001–3 mM) for 30 min at 37 °C. Lipid peroxidation was initiated by incubating the microsomes in the presence of 0.2 mM $FeCl_3$, 1.7 mM ADP and 0.2 mM NADPH for 20 min at 37 °C. The reaction was stopped by addition of EDTA (2 mM). In other experiments, microsomes were incubated with *N*-acetylserotonin (0.001–3mM) in the absence of reagents to cause lipid peroxidation. In all experiments, membrane fluidity and malondialdehyde plus 4-hydroxyalkenals levels were measured.

2.6. Statistical analysis

Data are presented as the means ± S.E.M. Student's *t*-test was performed for comparisons between means, and the level of significance was set at 0.05.

3. Results

Table 1 summarizes the membrane fluidity values and malondialdehyde plus 4-hydroxyalkenals concentrations measured in microsomes incubated in the presence (lipid peroxidation induced) and absence (control) of $FeCl_3$, ADP and NADPH. The oxidant reagents significantly raised malondialdehyde plus 4-hydroxyalkenals concentrations relative to control values, which indicate that membrane lipid peroxidation was produced. Oxidative damage to lipids was also associated with a reduction in membrane fluidity, as shown by the increase in the polarization parameter. Preincubation with *N*-acetylserotonin (0.001–3 mM) reduced these changes in a concentration-dependent

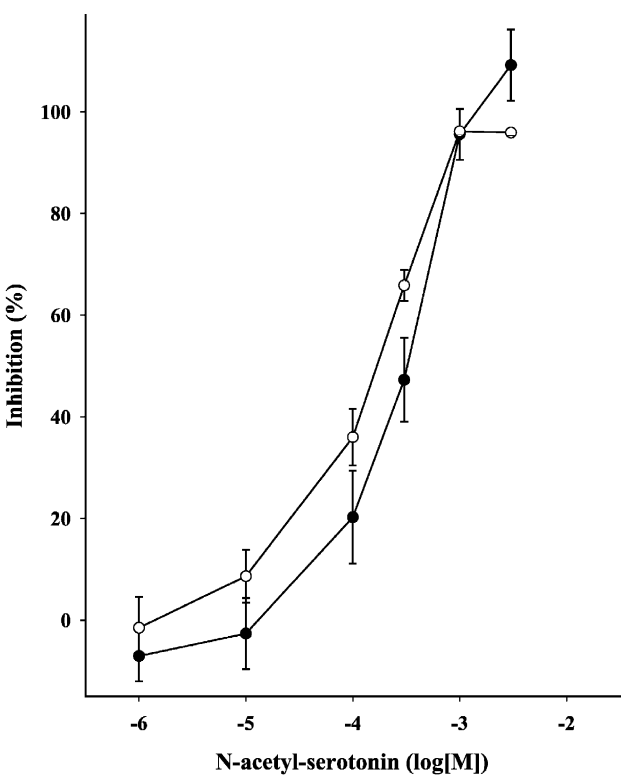

Fig. 2. *N*-acetylserotonin (0.001–3 mM) reduces rigidity in microsomal membranes due to lipid peroxidation. Data indicate its ability to prevent the decrease in microsomal membrane fluidity (●) and malondialdehyde plus 4-hydroxyalkenals formation (○). Percentages are expressed as means ± S.E.M. obtained in four independent experiments.

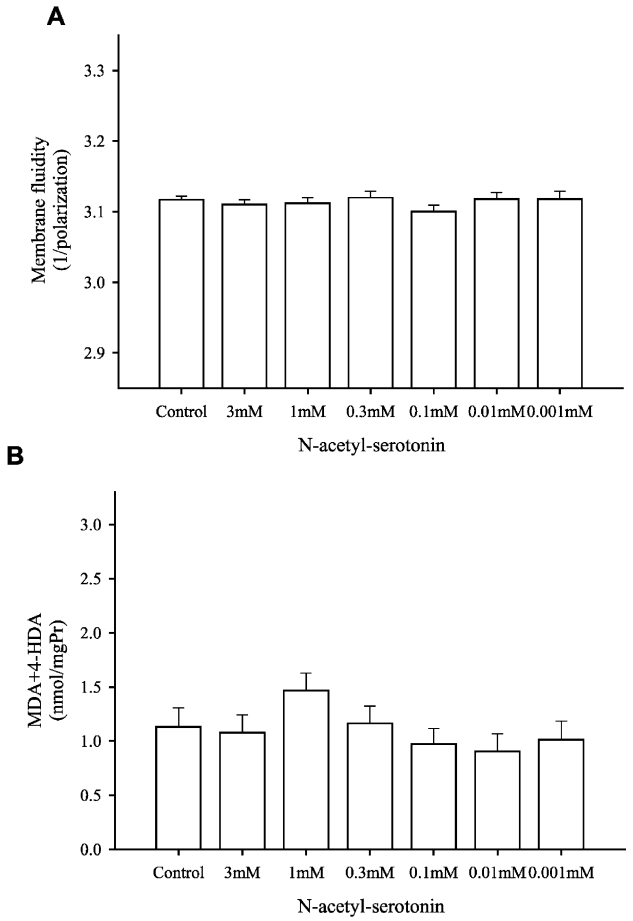

Fig. 3. Effects of *N*-acetylserotonin on membrane fluidity (A) and malondialdehyde plus 4-hydroxyalkenals concentrations (B) in hepatic microsomal membranes in the absence of oxidative stress. The values represent the means ± S.E.M. of four experiments.

manner. At 3 mM, *N*-acetylserotonin completely prevented the membrane rigidity and the lipid peroxidation.

The *N*-acetylserotonin concentration required to reduce the rigidity by 50% (IC_{50}) was 0.35 mM, slightly higher than that necessary for inhibiting malondehyde plus 4-hydroxyalkenals formation (0.19 mM). IC_{50} concentrations were calculated from the data presented in Fig. 2.

Fig. 3 illustrates the effect of *N*-acetylserotonin (0.001–3 mM) on membrane fluidity and malondehyde plus 4-hydroxyalkenals formation in the absence of lipid peroxidation. No significant changes were observed after the addition of *N*-acetylserotonin (0.001–3 mM) when they were compared to fluidity and malondehyde plus 4-hydroxyalkenals values in the absence of the drug.

4. Discussion

It is well documented that free radicals disturb phospholipid motion in the lipid bilayer. In our hands, the exposure of microsomal membranes to $FeCl_3$, ADP and NADPH induced lipid peroxidation, as indicated by larger increase in both malondehyde plus 4-hydroxyalkenals concentrations and polarization, reflecting membrane rigidity. These data are consistent with those reported elsewhere using a wide variety of biological systems to induce oxidative stress in membranes (Curtis et al., 1984; Watanabe et al., 1990; Viani et al., 1991; Yu et al., 1992; Choe et al., 1995). A possible explanation for the membrane rigidity is that free radicals, and consequently lipid peroxidation, modify the lipid composition of the membranes. After oxidative stress, a decrease in the membrane unsaturated/saturated fatty acid ratio is detected along with the formation of cross-linked lipid–lipid and lipid–protein moieties (Curtis et al., 1984; Levin et al., 1990; Martín-Valmaseda et al., 1999; Niranjan and Krishnakantha, 2000).

The maintenance of adequate membrane fluidity is essential for optimal cell physiology. Thus, there is increasing interest in molecules that are able to stabilize membranes against oxidative stress. We show herein that the indoleamine *N*-acetylserotonin protects against membrane rigidity induced by $FeCl_3$, ADP and NADPH in a concentration-dependent manner. Moreover, the protective effect of *N*-acetylserotonin on membrane lipid dynamics runs parallel to its ability to prevent malondehyde plus 4-hydroxyalkenals formation, suggesting that the mechanism responsible for stabilizing membranes is related to the antioxidant ability.

The antioxidant role of *N*-acetylserotonin is supported by in vitro studies which showed that the indoleamine reduced lipid peroxidation caused by iron, hydrogen peroxide and 2,2′-azobis(2-amidinopropane) (Chan and Tang, 1996; Longoni et al., 1997; Lezoualc'h et al., 1998; Siu et al., 1999), decreased oxidation of low-density lipoproteins due to CuSO4 (Seeger et al., 1997; Gozzo et al., 1999) counteracted t-butylated hydroperoxyde- and diamine-induced reactive oxygen species formation in lymphocytes (Wölfler et al., 1999), protected against DNA damage due to exposure to chromium (III) and hydrogen peroxide (Qi et al., 2000), and scavenged nitric oxide (Turjanski et al., 2001).

By contrast, little is known about the antioxidant ability of *N*-acetylserotonin in vivo. The administration of *N*-acetylserotonin to rats treated with α-naphthylisothiocyanate prevented malondehyde plus 4-hydroxyalkenals formation and membrane rigidity induced by the hepatotoxin. In this study, however, *N*-acetylserotonin failed to prevent the increases in hepatic enzymes and bilirubin levels in serum (Calvo et al., 2001).

It has been proposed that melatonin delays age-dependent free radical damage (Reiter, 1995). Lifelong pinealectomy aggravates membrane rigidity and increases several markers of oxidative damage, such as malonaldehyde and 8-hydroxy-deoxyguanosine levels, compared to those of intact age-matched control animals (Reiter et al., 1999). The data presented herein suggest that *N*-acetylserotonin, another pineal indoleamine that is reduced by pinealec-

tomy, may contribute to the suppression of membrane rigidity and lipid peroxidation due to oxidative stress in vitro. It is important to note that although a main product of the pineal gland is melatonin, this organ synthesizes other potentially active compounds. Besides *N*-acetylserotonin, four other tryptophan metabolites including melatonin (Calvo et al., 2001; García et al., 1997, 1998; Karbownik et al., 2000a,b), pinoline (García et al., 1999), 5-methoxytryptophol (García et al., 2000) and 6-hydroxymelatonin (Calvo et al., 2001) have similar abilities in stabilizing membranes both in vitro and in vivo. Moreover, in vitro evidence suggests that these compounds may act synergistically (Siu et al., 1999; García et al., 1999). Thus, it is possible that the antioxidant potential of the pineal may depend on the combined effect of several metabolites produced by the gland.

Under experimental conditions used herein, the IC_{50} values of *N*-acetylserotonin to reduce the malondehyde plus 4-hydroxyalkenals formation and membrane rigidity were higher than its physiological concentrations in blood. However, $FeCl_3$, ADP and NADPH levels used to induce lipid peroxidation were also much higher than their levels in biological fluids. Although physiological levels of *N*-acetylserotonin are reported to be antioxidative (Longoni et al., 1997), at the present time, only physiological concentrations of melatonin have been shown to contribute to the antioxidant capacity of human serum (Benot et al., 1999).

When compared to other indoles in stabilizing membranes, *N*-acetylserotonin was slightly more active than melatonin (García et al., 1997). The significance of this apparent difference remains unknown, however, caution is needed when interpreting these results since the role of *N*-acetylserotonin as an in vivo antioxidant remains essentially uninvestigated. Further studies are required to understand the relative contribution of *N*-acetylserotonin to the antioxidant defense system as well as its distribution, efficacy and toxicity when it is administrated in vivo.

We also showed in this report that *N*-acetylserotonin only improved membrane fluidity under conditions of induced oxidative stress. This is consistent with previous observations where melatonin also did not modify microsomal membrane fluidity in the absence of lipid peroxidation (Calvo et al., 2001; García et al., 1997; Costa et al., 1997; Karbownik et al., 2000a,b). In contrast, pinoline and 5-methoxytryptophol reduce fluidity in control membranes, although this effect only occurs in vitro at concentrations above 1 mM (García et al., 1999, 2000). This difference may be explained by the molecular structure of the molecules studied. *N*-acetylserotonin and melatonin differ only in the presence of either a hydroxy or a methoxy group in position 5 of the indole ring, respectively. Pinoline is a tricyclic metabolite of melatonin belonging to the carboline family; it is formed by condensation of an indoleamine with an aldehyde. In turn, 5-methoxytryptophol is a product of serotonin metabolism in which the indole ring is conserved but differs from *N*-acetylserotonin and melatonin in the composition of the *N*-acetyl chain. Non-indole antioxidants able to prevent membrane rigidity due to oxidative stress may be intercalated into the cell membrane disturbing its narrow physiological range of fluidity (Ohyashiki et al., 1986; Patel and Edwards, 1988; Dicko et al., 1999; Golden et al., 1999).

In conclusion, we report for the first time that pharmacological *N*-acetylserotonin concentrations prevent the rigidity of microsomal membranes caused by $FeCl_3$, ADP and NADPH. Based on our observations and previous reports on the antioxidant activity of *N*-acetylserotonin, the stabilizing effect of *N*-acetylserotonin in the membrane seems to depend on its ability to reduce lipid peroxidation. These findings add new information related to the protective role of pineal products against free radical toxicity.

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