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5-Methoxytryptophol Preserves Hepatic Microsomal Membrane Fluidity During Oxidative Stress

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Abstract Lipid peroxidation is a degenerative chain reaction in biological membranes that may be initiated by exposure to free radicals. This process is associated with changes in the membrane fluidity and loss of several cell membrane-dependent functions. 5-methoxytryptophol (ML) is an indole isolated from the mammalian pineal gland. The purpose of this study was to investigate the effects of ML (0.01mM-10mM) on membrane fluidity modulated by lipid peroxidation. Hepatic microsomes obtained from rats were incubated with or without ML (0.01–10 mM). Then lipid peroxidation was induced by $FeCl_3$, ADP, and NADPH. Membrane fluidity was determined using fluorescence spectroscopy. Malonaldehyde (MDA) +4-hydroxyalkenals (4-HDA) concentrations were estimated as an indicator of the degree of lipid peroxidation. With oxidative stress, membrane fluidity decreased and MDA+4-HDA levels increased. ML (0.01–3 mM) reduced membrane rigidity and the rise in MDA+4-HDA formation in a concentration-dependent manner. 10 mM ML protected against lipid peroxidation but failed to prevent the membrane rigidity. In the absence of oxidative reagents, ML (0.3–10 mM) decreased membrane fluidity whereas MDA+4-HDA levels remained unchanged. This indicates that ML may interact with membrane lipids. The results presented here suggest that ML may be another pineal indoleamine (in addition to melatonin) that resists membrane rigidity due to lipid peroxidation. J. Cell. Biochem. 76:651–657, 2000.

Key words: 5-methoxytryptophol; lipid peroxidation; membrane fluidity; microsome

Seven years after the isolation of melatonin from the bovine pineal gland [Lerner et al., 1958], McIsaac et al. [1965] detected the presence of another methoxyindole compound, 5-methoxytryptophol (ML), in this tissue. Subsequently, other workers also found ML in the pineal of several animal species [Skene et al., 1986, 1987; Lakhdar-Ghazal et al., 1992; Míguez, et al., 1996]. Biosynthesis of ML occurs from either serotonin or 5-methoxytryptamine and it is catalyzed by monoamine oxidase, alcohol dehydrogenase, and hydroxyindole-O-methyltransferase [McIsaac et al., 1965; Hardeland et al., 1993]. ML concentrations in the pineal gland show a daily rhythm, opposite that of melatonin; ML is found in highest concentrations during daytime and low concentration during the nighttime [Skene et al., 1987; Lakhdar-Ghazal et al., 1992; Míguez, et al., 1996; Zawilska et al., 1998]. Although ML seems less active than melatonin in terms of influencing reproductive physiology, it is reportedly involved in the modulation of some aspects of puberty and gonadal function [Reiter, 1980; Molina-Carballo et al., 1996].

Aerobic organisms generate free radicals as a consequence of the oxygen metabolism. Once formed, these unstable molecules react with neighboring macromolecules, which leads to structural modifications and loss in cellular organelle functions [Yu, 1994]. Polyunsaturated fatty acids, present in the membranes, are particularly sensitive to the aggressive behavior of free radicals [Rice-Evans and Burdon, 1993]. Both membrane structural intactness and adequate dynamics of the lipid bilayer are indispensable requisites for optimal functioning of cells [Stubbs and Smith, 1984]. Thus,

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lipid peroxidation and aberrant phospholipid motion in the membrane have been implicated in numerous degenerative diseases [Zubenko, 1986; Canuto et al., 1995]. Therefore, an interest in molecules that preserve the ideal fluidity of biological membranes because they neutralize free radicals is justified.

In recent years it has been demonstrated that several indoles, including ML, prevent oxidative damage [Poeggeler et al., 1996]. Melatonin, another indole produced in the pineal gland, scavenges free radicals, and moreover, it stimulates some enzymes involved in the antioxidant defense system [for review see Reiter, 1998a]. We have also demonstrated that melatonin stabilizes hepatic microsomal membranes induced by oxidative damage [García et al., 1997, 1998].

Because ML is synthesized and released from the pineal gland in a circadian rhythm with higher concentrations during light (as opposed to melatonin, which is high at night), and it also has antioxidant activity, the purpose of the present study was to investigate whether ML may also stabilize biological membranes against lipid peroxidation. In addition, we tested the effect of ML on the membranes in the absence of oxidative stress.

MATERIALS AND METHODS

Chemicals

ML, ferric chloride ($FeCl_3$), adenosine 5′-diphosphate (ADP), nicotinamide adenine dinucleotide phosphate (NADPH) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene-sulfonate (TMA-DPH) was from Molecular Probes. The Bioxytech LPO-586 kit for lipid peroxidation was obtained from Caymen Chemical (Ann Arbor, MI). Other chemicals used were of the highest quality available. ML was diluted in methanol and TMA-DPH in tetrahydrofuran and water. Methanol and tetrahydrofuran were 5% and 0.4% in the final mixture, respectively. $FeCl_3$, ADP, NADPH, and EDTA were diluted in the incubation buffer.

Animals and Membrane Preparation

Male Sprague-Dawley rats weighing 230g—250g were purchased from Harlan (Houston, TX). They were kept in a light:dark cycle of 14:10 and received standard diet and water ad libitum. Animals were maintained in accordance with the guidelines outlined by NIH and all procedures used were approved by the Institutional Animal Care and Utilization Committee. After being acclimated for two weeks, the animals were killed by decapitation and the liver was dissected, washed in saline solution (0.9% NaCl), frozen in liquid nitrogen, and stored at –70°C.

Hepatic microsomal membranes were isolated by the differential centrifugation method described by Yu et al. [1992]. Briefly, tissue was homogenized in 1/10 w/v in 140mM KCl/20mM HEPES buffer (pH = 7.4) and centrifuged at 1,000 x g for 10 min. The supernatant was centrifuged at 105,000 x g for 60 min. The pellet was re-suspended in the buffer and centrifuged at 10,000 x g for 15 min. Finally, the supernatant was re-centrifuged at 105,000 x g for 60 min and the resulting pellet suspended 1/1 v/v and stored at –70°C until use.

Peroxidation of Microsomes

Microsomal membranes (0.5 mg/ml) were suspended in 50mM Tris-HCl buffer (pH = 7.4) and were incubated with ML (0.01, 0.1, 0.3, 1, 3, and 10 mM) for 30 min at 37°C. Peroxidation of microsomes was induced by the addition of $FeCl_3$ (0.2mM), ADP (1.7mM) and NADPH (0.2mM) followed by incubation for 20 min at 37°C. The reaction was stopped by addition of EDTA (2mM). Microsomes with and without induced lipid peroxidation were exposed to the incubation as were those treated with ML. All solutions were prepared just before use in the assay.

Measurements of Fluidity in the Microsomal Membranes

Immediately after lipid peroxidation, fluorescence polarization studies were conducted to assess microsomal membrane fluidity [Yu et al., 1992]. In this method, a fluorescent molecule is incorporated into the membrane and emits a fluorescence signal after being exposed to polarized light [Zimmer et al., 1993]. Microsomal membranes were labeled using TMA-DPH. This probe is a DPH derivative, which incorporates a trimethylammonium substituent to improve its localization in the membrane. TMA-DPH is intercalated parallel to the long molecular axis of the phospholipids with the cationic residue oriented to the surface [Prendergast et al., 1981].

Aliquots of microsomal membranes containing 0.5 mg protein were suspended in 50 mM Tris-HCl buffer (pH = 7.4) (3 ml), mixed with

TMA-DPH (66.7 nM) and incubated at 37°C for 30 min. Fluorescence polarization was monitored in a Perkin-Elmer LS-50 Luminescence Spectrometer equipped with a circulator bath to maintain the temperature cuvette at 22±0.01°C. The probe was excited at 360 nm and its emission recorded at 430 nm. Polarization (P), an average of 30 observations for each sample, was calculated from observed relative fluorescence intensities of vertically polarized light detected by an analyzer oriented parallel (IV_V) or perpendicular (IV_H) to the excitation plane by the following equation:

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

G is a correction factor for the optical system.

The degree of polarization is a quantitative index of the freedom of rotation of TMA-DPH. A decrease in polarization indicates larger TMA-DPH mobility and therefore increased membrane fluidity. Thus, the results of membrane fluidity were expressed as the inverse of P [Yu et al., 1992; Garzetti et al., 1993].

MDA+4-HDA Measurements

Lipid peroxidation was measured by the formation of malonaldehyde (MDA) + 4-hydroxyalkenals (4-HDA). These products were determined by the above mentioned kit. In this assay, MDA and 4-HDA react with a chromogenic reagent, N-methyl-2-phenylindole, yielding a chromophore, which is measured at 586 nm. Results were expressed as nmol MDA+4-HDA/mg microsomal protein.

Protein Determination

Protein content was determined by the method of Bradford [1976] using serum albumin as standard.

Statistical Analysis

Results were expressed as means ± SEM. Statistical comparisons were performed using a one-way analysis of variance followed by Student-Newman-Keuls test with significance determined at a level of <0.05.

RESULTS

Effect of ML in Stabilizing Microsomal Membranes Against Oxidative Damage

The addition of $FeCl_3$, ADP, and NADPH to the incubation medium resulted in enhanced MDA+4-HDA concentrations. When TMA-DPH was incorporated into the microsomal membranes, fluorescence polarization intensity increased indicating a decrease in membrane fluidity.

The quantity of MDA+4-HDA produced under the conditions mentioned above was progressively reduced by treatment of the samples with ML. The concentration of ML required to inhibit MDA+4-HDA formation by 50%, i.e., IC_{50}, was 0.7 mM. Although less efficient in preventing MDA+4-HDA formation, ML (0.01–3mM) decreased membrane rigidity in a dose-dependent manner. In contrast, a 10 mM ML concentration failed to prevent microsomal membrane rigidity due to lipid peroxidation. Figure 1 summarizes these results.

Influence of ML on the Membrane Fluidity in Absence of Lipid Peroxidation

To examine the effect of ML in the absence of lipid peroxidation, microsomes were incubated with ML following the identical procedures, i.e., incubation conditions and ML concentrations, as in the previous experiments. Data shown in Figure 2A reflect clearly that ML (0.3–10 mM) caused a significant reduction in fluidity relative to the microsomes incubated without ML.

In contrast, no differences were detected in MDA+4-HDA levels after treatment with ML. Figure 2B summarizes the MDA+4-HDA concentrations after incubation with ML (0.01–10 mM).

DISCUSSION

Studies in a wide variety of membranes have demonstrated that free radicals disturb the order and lipid dynamics of the membrane [Curtis et al., 1984; Watanabe et al., 1990; Viani et al., 1991; Dinis et al., 1993]. Order in the membrane is a term that refers to the mean angular deviation from the bilayer plane of the fatty acid chain and it is inversely related to the fluidity of the membrane [Curtis et al., 1984]. Two primary reasons have been proposed to explain loss in the membrane fluidity due to lipid peroxidation. First the reduction in mem-

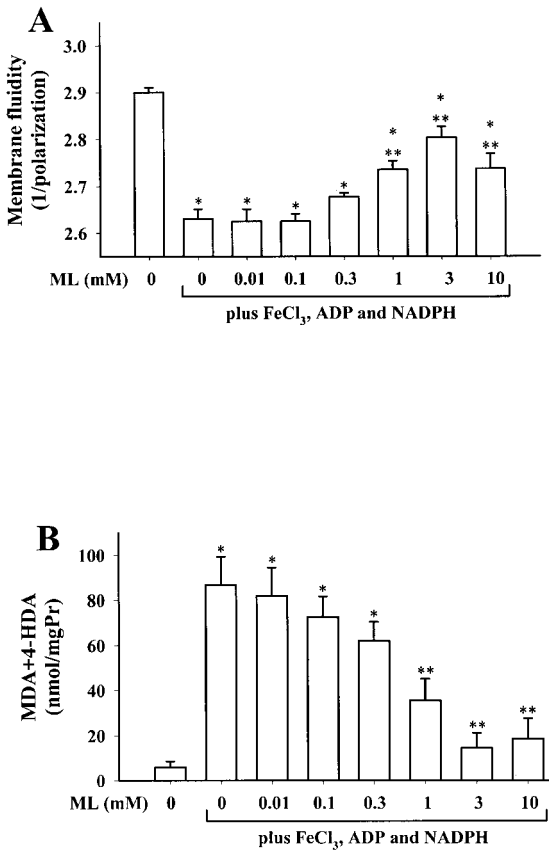

Fig. 1. **A:** Effect of 5-methoxytryptophol (0.01mM-10mM) on microsomal membrane fluidity and **B:** MDA+4-HDA concentrations after oxidative damage induced by exposure to 0.2mM $FeCl_3$, 1.7mM ADP, and 0.2mM NADPH. Each value represents the mean ± standard errors obtained in four independent experiments. $^*P < 0.05$ compared to the samples treated without oxidative stress. $^{**}P < 0.05$ compared to microsomes exposed to $FeCl_3$, ADP, and NADPH alone.

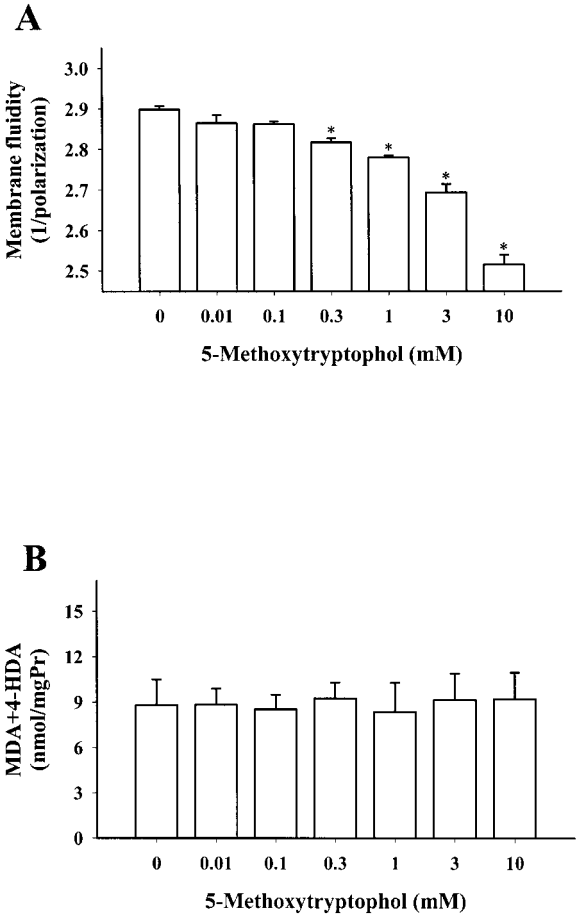

Fig. 2. **A:** Effects of 5-methoxytryptophol on microsomal membrane fluidity and **B:** MDA+4-HDA concentrations in the absence of oxidative stress. The values represent the mean ± standard errors of four experiments. $^*P \leq 0.05$ versus microsomes without exposure to 5-methoxytryptophol.

brane polyunsaturated fatty acid concentrations [Curtis et al., 1984] and, second, the cross linking between adjacent molecules may account for the alterations in membrane fluidity [Eichenberger et al., 1982; Chen and Yu,1994]. Our data obtained in hepatic microsomes are consistent with these observations. Incubation of microsomal membranes with $FeCl_3$, ADP, and NADPH was followed by MDA+4-HDA accumulation, an index of lipid peroxidation, and a loss in membrane fluidity.

The addition of ML (0.1–3 mM) prevented both the increase in MDA+4-HDA levels and the membrane rigidity. Recent data have shown that ML, melatonin, and other methoxylated indoles act as radical scavengers as indicated by their ability to resist the oxidation of a radical trapping reagent, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) [Poeggeler et al., 1996]. Tan et al. [1993, 1998] have uncovered the mechanisms by which melatonin scavenges the hydroxyl radical, and Pieri et al. [1994] have suggested that melatonin detoxifies the peroxyl radical. Previous studies conducted in our laboratory have shown that melatonin stabilizes membranes and that this effect relates to its free radical scavenging activity [García et al., 1997, 1998]. Additionally, pinoline, another methoxylated metabolite of the essential amino acid tryptophan, also reduces both lipid peroxidation in brain homogenates [Pless et al., 1999] and hepatic microsomal rigidity due to free radical exposure [unpublished observations]. Because ML reduced MDA+4-HDA formation, it seems possible that under our experimental

conditions the mechanism by which ML preserved microsomal membrane fluidity was related to its prevention of lipid peroxidation.

Pineal gland failure and a reduction in melatonin have been associated with aging [Reiter, 1998b]. Age-dependent changes in biological membranes include decreases in the membrane fluidity due to an increase of cholesterol content in the membrane and increased levels of lipid peroxidation products [Vorbeck et al., 1982; Cohen and Zubenko, 1985; Choe et al., 1995; Choi and Yu, 1995]. Our findings, although obtained using pharmacological concentrations of ML, may be consistent with the decreases observed in hepatic microsomal membrane fluidity in pinealectomized old rats compared to age-matched control animals [Reiter et al., 1999]. Since ML, melatonin and pinoline have membrane-stabilizing abilities, it seems possible that the drop in their concentrations may be partially responsible for increased cell membrane rigidity in old animals. Further studies are required to determine the relative importance of life-long changes in ML and pinoline concentrations during aging.

In opposition to the scavenging hypothesis presented to explain how ML preserves the fluidity of the membranes modulated by lipid peroxidation, 10mM ML efficiently reduced lipid peroxidation but failed to prevent microsomal membrane rigidity (Fig. 1). The reason for this unexpected divergence may be explained by the experiment designed to investigate the effect of ML on the membrane in the absence of oxidative damage. ML (0.3–10 mM) caused a pronounced increase in the fluorescence polarization indicating that membrane fluidity decreased without a modification of MDA+4-HDA levels (Fig. 2). These data suggest that the acyl lipid mobility was reduced by ML at least in the region wherein the probe was located. In addition, this may explain the lower efficacy of ML in preserving the rigidity compared to its antioxidant ability.

Other antioxidants that are effective in preventing membrane rigidity due to lipid peroxidation may also disturb lipid motion in the membrane without oxidative stress. Kaplán et al. [1995] have shown that α-tocopherol, a lipid soluble dietary antioxidant, prevents changes in brain endoplasmic reticulum membranes due to oxygen-free radicals; however, Massey et al. [1982] found that α-tocopherol perturbs lipid packing in liposomes in the absence of oxidative damage. We have demonstrated that tamoxifen, an antiestrogenic drug used in breast cancer treatment, also prevents microsomal membrane rigidity induced by lipid peroxidation, an effect enhanced by melatonin, because melatonin may possibly reduce the IC_{50} concentration of tamoxifen [García et al., 1998]. Tamoxifen also may increase fluorescence anisotropy, which represents a decrease in the fluidity of liposomal membranes [Wiseman et al., 1993].

In comparing the membrane stabilizing effect of ML with other pineal products, it was found that pinoline, although more active that ML in preventing microsomal rigidity due to oxidative stress, also altered membrane fluidity when the concentrations were higher than 1mM (unpublished observations). In contrast to ML and pinoline, melatonin reduces the rigidity of microsomal membranes exposed to an identical oxidant model that was used herein to the levels that matched those of control membranes, without altering the fluidity, in absence of lipid peroxidation [García et al., 1997].

It may be pertinent to note that the effects of ML on the membrane under basal levels of oxidative stress occur at much higher concentrations than are detected in the pineal gland [Skene et al., 1987; Lakhdar-Ghazal et al., 1992; Míguez, et al., 1996; Zawilska et al., 1998] and thus we suggest that, at physiological concentrations, this side effect would likely not be observed. However, since membrane fluidity modulates numerous functions of the cell, it may be necessary to limit ML use as a therapeutic antioxidant.

It seems reasonable to conclude that pharmacological concentrations of ML protected microsomal membranes against the rigidity induced by the addition of $FeCl_3$, ADP, and NADPH. We suggest that, under lower oxidative levels, stabilizing membranes may be another mechanism by which ML protects cells. Considering the previous results obtained from other tryptophan metabolites, the data are consistent with the idea that secretory products of the pineal gland may play a role in stabilizing biological membranes and this may be related to the antioxidant properties of these products.

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