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Effects of Tryptophan and 5-Hydroxytryptophan on the Hepatic Cell Membrane Rigidity Due to Oxidative Stress

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Abstract The ability of several indoleamines to scavenge free radicals is well documented. Our aim was to evaluate the ability of 0.01–3 mM tryptophan (Trp) and 0.1–5 mM 5-hydroxytryptophan (5-OH-Trp) to protect hepatic cell membranes against 0.1 mM $FeCl_3$ plus 0.1 mM ascorbic acid–induced lipid peroxidation and increases in membrane rigidity. Membrane fluidity was evaluated using fluorescence spectroscopy. Lipid and protein oxidation were estimated by quantifying malondialdehyde (MDA) plus 4-hydroxyalkenals (4-HDA) concentrations and carbonyl group content, respectively. Exposure to $FeCl_3$ plus ascorbic acid increased hepatic cell membrane rigidity, MDA + 4-HDA and carbonyl content. The presence of 5-OH-Trp, but not Trp, attenuated these changes. In the absence of oxidative stress, neither indoleamine modified fluidity, MDA + 4-HDA or carbonylation. These results suggest that C5 hydroxylation determines the ability of Trp to preserve membrane fluidity in the presence of oxidative stress.

Keywords Tryptophan · 5-Hydroxytryptophan · Membrane fluidity · Lipid peroxidation · Protein oxidation · Antioxidant

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Introduction

Tryptophan (Trp) is an essential amino acid that is a constituent of biological membranes and contributes to the solubility of membrane proteins (Schiffer et al. 1992). The 5-hydroxy derivative of Trp, 5-OH-Trp, is a popular dietary supplement used in the treatment of depression, fibromyalgia, obesity, hypertension, insomnia and headaches (Das et al. 2004). It is biosynthesized from Trp by Trp-5-hydroxylase in the pineal gland of many animal species (Das et al. 2004). 5-OH-Trp is a precursor of melatonin, an indoleamine with a wide range of antioxidant abilities (Ghosh et al. 2007; Mollaoglu et al. 2007; Ortega-Gutiérrez et al. 2007; Reiter et al. 2007; Saravanan et al. 2007).

Aerobic organisms generate free radicals as a consequence of oxygen metabolism. Once formed, these electronically unstable chemical species can react with neighboring biomolecules (García et al. 2000; Yu 1994). Peroxidation of the polyunsaturated fatty acids in cell membranes is a degenerative chain reaction initiated by exposure to free radicals. This process leads to formation of the peroxyl radical ($LOO^{\cdot}$), endoperoxides and hydroperoxides (Curtis et al. 1984). $LOO^{\cdot}$ radicals are sufficiently powerful to propagate lipid peroxidation in adjacent healthy phospholipids (Maiorino et al. 1989). Perhaps the most dangerous functional consequence of lipid peroxidation is the disruption of membrane fluidity; optimal fluidity is mandatory for various essential cell functions including solute transport, signal transduction and the activity of enzymes associated with the cell membranes (Curtis et al. 1984; García et al. 1997; Stubbs and Smith 1984; Van Blitterswijk 1985). Several antioxidant molecules, including the pineal indoleamines melatonin (García et al. 1997), *N*-acetyl-serotonin (Calvo et al. 2001; García et al. 2001; Karbownik et al. 2001), 5-methoxytryptophol (García et al.

2000) and pinoline (Ortega-Gutiérrez et al. 2002), have been described as protectors of cell and organelle membranes because they inhibit lipid peroxidation and prevent membrane rigidity. Because Trp and 5-OH-Trp are the chemical precursors of these indoleamines, we evaluated the ability of these amino acids to preserve membrane fluidity and protect lipids and proteins against oxidative stress caused by exposure to $FeCl_3$ plus ascorbic acid.

Materials and Methods

Chemicals

Trp, 5-OH-Trp, $FeCl_3$, ascorbic acid, ethylenediaminetetraacetic acid disodium (EDTA-Na_2), Tris(hydroxymethyl) aminomethane (TRIS) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma–Aldrich (Madrid, Spain). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene-sulfonate (TMA-DPH) was obtained from Molecular Probes (Eugene, OR). Other chemicals were of analytical grade and were purchased from commercial sources. TMA-DPH was diluted in tetrahydrofuran (THF) and water. THF concentration in the incubation reaction was 0.4%. Trp, 5-OH-Trp, $FeCl_3$ and ascorbic acid were diluted in 50 mM Tris–HCl (pH 7.4) and freshly prepared prior to use.

Animals and Membrane Isolation

Animal handling and procedures were performed in strict accordance with the recommendations of the Committee of the European Union (86/609/CEE) for the care and use of laboratory animals. Male Sprague–Dawley rats (Harland Ibérica, Barcelona, Spain) weighing 200–225 g were anesthetized with 50 mg/kg thiopental and subjected to intracardiac perfusion with cold saline solution. The livers were removed quickly, washed in saline, frozen and stored at −80°C until use. Pooled membranes were isolated according to Yu et al. (1992). Briefly, livers were homogenized 1:10 (w/v) in 140 mM KCl/20 mM HEPES buffer (pH 7.4) and then centrifuged at 1,000×*g* for 10 min at 4°C. The supernatant was centrifuged at 50,000×*g* for 20 min at 4°C. The pellet was resuspended in the same buffer and centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant and the buffy coat were homogenized and centrifuged at 50,000×*g* for 20 min at 4°C. The final pellet was resuspended 1:1 w/v and stored at −80°C until assay.

Peroxidation of Membranes

Hepatic cell membranes suspended in 50 mM TRIS–HCl (0.5 mg membrane protein/ml) were incubated with Trp (0.01, 0.1, 0.3, 1.0 or 3.0 mM) or 5-OH-Trp (0.1, 0.3, 1.0, 3.0 or 5.0 mM) at 37°C for 120 min. Lipid oxidation and protein oxidation were induced by adding 0.1 mM $FeCl_3$ plus 0.1 mM ascorbic acid. The mixtures were shaken during incubation. The reaction was stopped by adding 2 mM EDTA-Na_2. Kinetic studies were performed to determine the optimal incubation times. Experiments were performed for control membranes (no oxidative stress and no indoleamines), membranes subjected to oxidative stress alone, membranes treated with Trp or 5-OH-Trp and subjected to oxidative stress and membranes treated with Trp or 5-OH-Trp without oxidative stress. Membrane fluidity, malondialdehyde (MDA) plus 4-hydroxyalkenal (4-HDA) concentrations and carbonyl group content were measured immediately after completion of treatments.

Analytical Procedures

Fluidity of hepatic cell membranes was monitored using TMA-DPH as a probe. The incorporation of TMA-DPH into the bilayer and membrane fluidity measurements were carried out according to the method of Yu et al. (1992). Briefly, 0.5 mg membrane protein/ml were suspended in 50 mM TRIS (3 ml final volume) and TMA-DPH (66.7 nM) was added. After vigorous mixing, the membrane suspensions were incubated at 37°C for 30 min. Fluorescence measurements (excitation $\lambda = 360$ nm, emission $\lambda = 430$ nm) were performed in a Perkin-Elmer (Oak Brook, IL) LS-55 Luminescence Spectrometer equipped with a circulating water bath to maintain a cuvette temperature of 22 ± 0.1°C. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel (I_{Vv}) or perpendicular (I_{VH}) to the excitation plane. A correction factor for the optical system, G, was used. Polarization (P) was calculated according to the equation

$$P = \frac{I_{Vv} - GI_{VH}}{I_{Vv} + GI_{VH}}$$

Because there is an inverse relationship between membrane fluidity and P (García et al. 1997, 2000; Lysek et al. 2003; Rice-Evans and Burdon 1993; Yu 1994; Yu et al. 1992), fluidity was expressed as 1/P and it was calculated from triplicate determinations. Protein concentrations were determined according to the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

The combined levels of MDA and 4-HDA (MDA + 4-HDA) was used as an index of lipid peroxidation in the hepatic cell membranes and was determined using a commercial colorimetric assay (Calbiochem, San Diego, CA) (Janero 1990). In this assay, MDA and 4-HDA react with *N*-methyl-2-phenylindole to yield a chromophore with peak absorbance at 586 nm; 1,1,3,3-tetramethoxypropane was used as standard. Results are expressed as nanomoles

of MDA + 4-HDA per milligram of protein. Carbonyl content, an index of oxidative protein damage, was measured according to Levine et al. (1990, 1994). In the assay, carbonyl groups interact with 2,4-dinitrophenylhydrazine to yield a stable complex with maximal absorbance at 375 nm. Results are expressed as nanomoles of carbonyl group per milligram of protein.

Statistical Analysis

Results are expressed as arithmetic mean ± standard error. Statistical comparisons were performed using Student's paired *t*-test with a level of significance of $P < 0.05$.

Results

Effects of Trp and 5-OH-Trp on Oxidative Damage-Induced Decreases in Membrane Fluidity

Incubating hepatic cell membranes with $FeCl_3$ plus ascorbic acid resulted in reduced membrane fluidity and the oxidation of membrane lipids and proteins, as shown by elevations in MDA + 4-HDA and carbonyl group concentrations, respectively (Table 1). Although including Trp in the incubation mixture failed to protect the membranes in terms of fluidity and biochemical markers of oxidative stress, inclusion of 0.3–5 mM 5-OH-Trp prevented the increases in membrane rigidity and elevations in MDA + 4-HDA content and protein carbonylation in a concentration-dependent manner (Table 1). The induction of membrane rigidity was inhibited completely when the concentration of 5-OH-Trp in the mixture was 5 mM. The concentrations of 5-OH-Trp required to inhibit membrane rigidity, the formation of MDA + 4-HDA and protein carbonylation by 50% (IC_{50}) were 2.40, 1.38 and 2.62 mM, respectively. Values were calculated using Fig. 1.

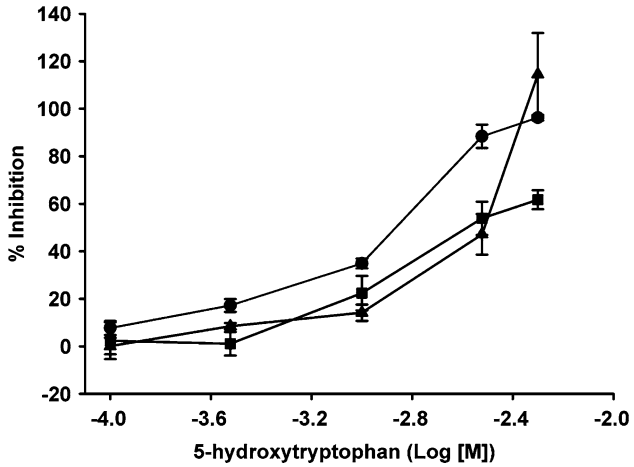

Fig. 1 Ability of 5-OH-Trp to reduce hepatic cell membrane rigidity (*filled triangle*), lipid peroxidation expressed as MDA + 4-HDA formation (*filled circle*) and protein oxidation (*filled square*). Percentages are expressed as means ± standard errors obtained from five independent experiments

Effects of Trp and 5-OH-Trp on Membrane Fluidity and MDA + 4-HDA and Carbonyl Group Concentrations in the Absence of Oxidative Stress

Table 2 summarizes the effects of Trp (0.01–3 mM) and 5-OH-Trp (0.1–5 mM) on membrane fluidity in the absence of oxidative stress. The polarization parameter P, which is inversely related to lipid bilayer fluidity, was minimally affected by incubation with the indoleamines, indicating

Table 1 Membrane fluidity, MDA + 4-HDA concentration and carbonyl group concentration after incubation with Trp or 5-OH-Trp in the presence or absence of lipid peroxidation (LPO)

	Membrane fluidity (1/polarization)		MDA + 4-HDA (nmol/mg protein)		Carbonyl group (nmol/mg protein)	
	Trp	5-OH-Trp	Trp	5-OH-Trp	Trp	5-OH-Trp
Control	3.70 ± 0.04[b]	3.62 ± 0.03[b]	1.36 ± 0.58[b]	0.95 ± 0.22[b]	5.30 ± 0.42[b]	6.17 ± 1.47[b]
LPO	3.37 ± 0.04[a]	3.24 ± 0.02[a]	37.99 ± 5.60[a]	35.21 ± 2.63[a]	22.56 ± 0.92[a]	29.19 ± 1.49[a]
LPO + indoleamine (mM)						
5	–	3.67 ± 0.09[b]	–	2.26 ± 0.36[b]	–	14.77 ± 0.96[a,b]
3	3.37 ± 0.02[a]	3.42 ± 0.06[a,b]	40.61 ± 4.74[a]	5.04 ± 1.69[a,b]	23.97 ± 0.73[a]	17.27 ± 1.86[a,b]
1	3.37 ± 0.04[a]	3.29 ± 0.03[a,b]	42.14 ± 4.98[a]	23.32 ± 1.98[a,b]	23.89 ± 2.78[a]	24.44 ± 2.28[a,b]
0.3	3.36 ± 0.03[a]	3.27 ± 0.02[a,b]	40.21 ± 5.24[a]	29.52 ± 2.78[a,b]	22.08 ± 1.19[a]	29.09 ± 1.90[a]
0.1	3.35 ± 0.03[a]	3.24 ± 0.03[a]	40.32 ± 4.76[a]	32.72 ± 2.97[a]	21.18 ± 1.90[a]	29.14 ± 2.50[a]
0.01	3.34 ± 0.03[a]	–	39.70 ± 5.12[a]	–	17.47 ± 1.84[a]	–

Results are reported as means ± standard errors of five experiments. Statistically significant differences ($P < 0.05$) [a] vs. control and [b] vs. $FeCl_3$ plus ascorbic acid

Table 2 Effects of Trp and 5-OH-Trp on membrane fluidity, MDA + 4-HDA concentration and protein carbonylation in hepatic cell membranes in the absence of oxidative stress (means ± standard errors of five experiments)

	Membrane fluidity (1/polarization)		MDA + 4-HDA (nmol/mg protein)		Carbonyl group (nmol/mg protein)	
	Trp	5-OH-Trp	Trp	5-OH-Trp	Trp	5-OH-Trp
Control	3.71 ± 0.05	3.62 ± 0.05	0.74 ± 0.42	1.03 ± 0.14	4.73 ± 0.75	3.56 ± 0.37
Indoleamine (mM)						
5	–	3.62 ± 0.03	–	0.99 ± 0.25	–	5.81 ± 0.74
3	3.75 ± 0.05	3.61 ± 0.04	1.54 ± 1.45	1.06 ± 0.22	6.31 ± 0.89	6.56 ± 1.08
1	3.74 ± 0.07	3.55 ± 0.04	0.91 ± 0.61	0.96 ± 0.17	6.75 ± 1.37	7.08 ± 0.90
0.3	3.76 ± 0.06	3.57 ± 0.04	1.02 ± 0.71	0.96 ± 0.12	7.66 ± 1.19	7.63 ± 0.90
0.1	3.71 ± 0.05	3.56 ± 0.02	1.18 ± 0.90	0.84 ± 0.10	6.31 ± 0.94	6.35 ± 0.68
0.01	3.74 ± 0.05	–	0.85 ± 0.53	–	6.50 ± 1.14	–

that they did not influence membrane fluidity under these conditions. Similarly, the concentrations of MDA + 4-HDA and carbonyl group in membranes incubated with either Trp (0.01–3 mM) or 5-OH-Trp (0.1–5 mM) were similar to those of membranes incubated without Trp or 5-OH-Trp (Table 2).

Discussion

The in vitro model that combines $FeCl_3$ plus ascorbic acid to induce oxidative stress in biomembranes is widely accepted in the field of oxidative stress research (Cadenas et al. 1989; Gavazza and Catala 2003; Ghosh et al. 1993; Guajardo et al. 2003; Millán-Plano et al. 2003). Under the experimental conditions used in our study, the exposure of isolated hepatic cell membranes to $FeCl_3$ plus ascorbic acid induced lipid and protein oxidation as indicated by enhanced MDA + 4-HDA concentration and increased protein carbonylation, respectively. The peroxidation of lipids within biological membranes impairs the function of proteins located in the membrane environment and changes the physicochemical properties of membranes, including membrane fluidity (García et al. 1997, 2000; Ghosh et al. 1993; Kaplan et al. 1995; Watanabe et al. 1990; Yu et al. 1992). In the present study, coincubation of hepatic cell membranes with $FeCl_3$ plus ascorbic acid resulted in a decrease in membrane fluidity. It is well documented that free radical-induced oxidative stress inhibits the mobility of lipids within membranes. The mechanisms by which this increased membrane rigidity can be explained are related to changes in the structure of the lipid bilayer, including a loss of polyunsaturated fatty acids (Bruch and Thayer 1983; Curtis et al. 1984) and cross-linking between lipid–lipid and lipid–protein moieties (Bruch and Thayer 1983; Eichenberger et al. 1982; Schroeder 1984).

In a previous study, we demonstrated that the endogenous antioxidant melatonin, the main derivative of Trp, stabilized hepatic microsomal membranes due to its ability to scavenge free radicals (García et al. 1997). Recent interest has focused on the localization of melatonin within the lipid bilayer to justify, at least in part, its ability to prevent membrane lipid peroxidation (de Lima et al. 2007). However, results concerning the antioxidant properties of Trp itself are conflicting. Some in vivo studies have demonstrated that Trp reduces edema and lipid peroxidation in cerulein- or ischemia/reperfusion-induced pancreatitis (Jaworek et al. 2003; Leja-Szpak et al. 2004) and accelerates healing of acute gastric ulcers induced by local administration of irritants including acetic acid, ethanol and aspirin (Brzozowska et al. 2002). Moreover, Trp showed weak antioxidant activity by scavenging nitric oxide, as examined by the Greiss reaction using flow injection analysis (Noda et al. 1999). On the other hand, Trp has been shown to promote oxidative stress. In rats fed Trp-supplemented diets, Trp appeared to enhance lipid peroxidation in plasma (Aviram et al. 1991) and induce damage to the eye lens (Mathur and Sahai 1990). In addition, in vitro evidence indicates that Trp increases lipid peroxidation and decreases antioxidant defences in the cerebral cortex of rats, which could partially explain the neurotoxic mechanisms of brain injury induced by hypertryptophanemia (Feksa et al. 2006). According to our results, Trp does not inhibit lipid and protein oxidation, as shown by its inability to prevent $FeCl_3$ plus ascorbic acid–induced decreases in membrane fluidity, increases in MDA + 4-HDA content and increases in protein carbonylation.

However, under identical oxidative stress-inducing conditions, the hydroxylated form of Trp reduced MDA + 4-HDA generation in a concentration-dependent manner. This is in accordance with the results of two previous studies showing a significant reduction in lipid peroxidation

in hepatic rat microsomes treated with Fe (Cadenas et al. 1989) and in phosphatidylcholine liposomes exposed to 2,2′-azobis(2amidinopropane) hydrochloride, a water-soluble azo-compound that generates peroxyl radicals (Christen et al. 1990). When the efficacies of 5-OH-Trp and melatonin were compared in terms of their ability to scavenge nitric oxide and to reduce the hyperglycemia-induced oxidative stress in kidney-cortex tubular cells, melatonin proved more effective (Noda et al. 1999; Derlacz et al. 2007). One reasonable explanation for the protective effect of 5-OH-Trp against lipid peroxidation is its ability to neutralize free radicals in the membranes (Keithahn and Lerchl 2005). Herein, we have extended the evidence of 5-OH-Trp as an antioxidant in terms of protein oxidation. Exposing membrane proteins to oxidative stress leads to gross structural and functional modifications, including protein fragmentation and aggregation, protein peroxide formation and enzyme inactivation (Mayo et al. 2003). 5-OH-Trp differs from Trp only in the substitution of a hydroxyl group for a hydrogen atom in position 5 of the indole ring. Although our study did not address the chemical mechanism of the antioxidant activity, the fact that the incorporation of the hydroxyl group into the amino acid activates its antioxidant behavior suggests that the protective effect of 5-OH-Trp is related to its ability to transfer electrons to neighboring free radicals.

To our knowledge, this study is the first to report that 5-OH-Trp stabilizes membranes against oxidative stress. The close concentration-dependent relationship between chemical membrane damage, expressed by MDA + 4-HDA formation and protein carbonylation, and the severity of membrane rigidity (Fig. 1) suggests that a radical scavenging mechanism is responsible for the stabilizing effect of 5-OH-Trp on hepatic membranes. Maintaining optimal biological membrane fluidity is crucial for numerous cell functions. Even slight changes in lipid bilayer fluidity can cause aberrant function and induce pathology (Van Blitterswijk 1985). Therefore, there is considerable interest in the identification of molecules that limit increases in membrane rigidity due to oxidative stress.

Melatonin is the major indoleamine synthesized from Trp in living beings in terms of cellular protection and antioxidant activity (Reiter et al. 2008). Several structurally related indoles, including 5-methoxytryptophol, *N*-acetylserotonin and a β-carboline formed by condensation between indoleamines and aldehydes, pinoline, may also act as powerful radical scavengers while stabilizing membranes (García et al. 1999, 2001). In addition, several endogenous kynuramines can neutralize free radicals (Leon et al. 2006; Ressmeyer et al. 2003) and therefore may help to preserve membrane fluidity by protecting against oxidative stress.

Although molecular modeling simulations have suggested electrostatic interactions between Trp and lipid bilayers (Norman and Nymeyer 2006), the mechanisms by which Trp might modify lipid dynamics in biological membranes are poorly understood. The lack of significant changes in polarization on exposing membranes to 5-OH-Trp in the absence of pro-oxidative reagents agrees with previous observations for melatonin and *N*-acetylserotonin (García et al. 1997, 2001). These endogenous indoleamines were only able to improve membrane fluidity under conditions of induced oxidative stress. On the other hand, several reports have claimed that other well-known antioxidants, such as tocopherols (Ohki et al. 1984; Ohyashiki et al. 1986) and tamoxifen (Wiseman et al. 1993), could modify membrane fluidity in the absence of oxidative stress. The fact that 5-OH-Trp did not modify membrane fluidity under basal conditions supports the hypothesis that free radical scavenging is the mechanism by which 5-OH-Trp stabilizes biological membranes.

In conclusion, these data demonstrate that the incorporation of a hydroxyl group in position 5 of the indole ring of Trp enables Trp to behave as an antioxidant. 5-OH-Trp protects against $FeCl_3$ plus ascorbic acid-induced increases in membrane rigidity, most likely due to its ability to inhibit lipid and protein oxidation. The results suggest that 5-OH-Trp may be another pineal molecule that prevents membrane rigidity mediated by lipid peroxidation.

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