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ORIGINAL ARTICLE

Melatonin prevents oxidative damage to protein and lipid induced by ascorbate-Fe^{3+}-EDTA: Comparison with glutathione and α-tocopherol

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Abstract

OBJECTIVES: The ability of melatonin to protect protein and lipid against oxidative damage induced by an ascorbate-Fe^{3+}-EDTA (AFE) system which generates the hydroxyl radical was investigated using bovine serum albumin (BSA) and phosphatidylcholine (PC) liposomes, respectively, and compared with the protective effects of reduced glutathione and α-tocopherol. The comparison study was also performed using PC liposomes containing BSA. METHODS: BSA, PC liposomes or their mixtures were exposed to the HO•-generating system of AFE composed of 0.1 mM EDTA-Fe^{3+} and 0.5 mM ascorbate in 0.1 M phosphate buffer, pH 7.4, at 37°C for 1 h. Oxidative damage of BSA was determined by measuring the carbonyl content and the fragmentation of protein by the reaction with dinitrophenylhydrazine (DNPH) and electrophoresis, respectively. Lipid peroxidation of PC liposomes was indicated by the quantity of malondialdehyde and 4-hydroxyalkenals. RESULTS: Melatonin inhibited protein damage as indicated by the reduced formation of carbonyl groups and fragmentation of BSA by AFE as effectively as did glutathione while α-tocopherol was ineffective. Melatonin also prevented lipid peroxidation to the same extent as did α-tocopherol in PC liposomes. CONCLUSION: Both BSA and PC lipid exposed to AFE are effectively protected by melatonin while hydrophilic glutathione and hydrophobic α-tocopherol are as effective as melatonin only in one target, i.e., BSA or PC lipid, respectively.

Introduction

Melatonin, a pineal secretory product related to annual fluctuations in reproductive competence due to seasonally changing photoperiods, to circadian rhythms, sleep mechanisms, immune function, cancer, and mood [1–6] has become of increasing interest since it was reported to be a scavenger of the highly toxic hydroxyl radical (HO•) [7, 8], to a limited degree the peroxyl radical (ROO•) [9–12], singlet oxygen (^1O) [13, 14] and the peroxynitrite anion ($ONOO^-$) [15]. Because of these reports, melatonin has been tested for its ability to resist oxidative damage induced by various means. The overwhelming majority of these studies have emphasized melatonin's ability to resist oxidative damage to lipid and DNA [16]. To date, however, there are few studies on its ability to protect proteins, although they are a primary site of oxidative damage [17].

Protein attack by radicals is estimated by investigating the formation of carbonyl groups, protein-protein cross-linkages, fragmentation, the oxidation of side chains and is increased in proteolytic susceptibility [18–20]. Carbonyl groups are a frequent consequence of protein oxidation and these are often accepted as markers of oxidative modification [21, 22]. Protein carbonyls are elevated in cell cultures and in animals following their exposure to various conditions of oxidative stress such as hyperoxia, forced exercise, ischemia-reperfusion, paraquat, ozone, cigarette smoke, gamma radiation or mixed function oxidation. They can also be produced in cell-free systems [18, 23]. Finally, protein carbonyls have been associated with a number of diseases including amyotrophic lateral sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, Werner's syndrome, pancreatitis and aging [24–26].

In the current study, the ability of melatonin to protect protein against reactive oxygen species was investigated using bovine serum albumin (BSA) and an ascorbate-Fe^{3+}-EDTA (AFE) system as a model of HO•-generating system; for this we measured the protein carbonyl content and also fragmentation. Melatonin's capacity to protect against protein damage was compared with that of other well known antioxidants, reduced glutathione and α-tocopherol.

In addition to its radical scavenging ability, melatonin has the unusual property of being highly lipid-soluble [27] while being somewhat water-soluble as well [28]. These features allow melatonin to readily cross all morphophysiological barriers and to distribute quickly to all tissues in both the lipid and aqueous portion of cells, a feature not shared by most antioxidants [29, 30]. In order to investigate the potential beneficial effects of this property of melatonin, melatonin's ability to protect both proteins in the hydrophilic phase and lipids in the hydrophobic phase was compared with that of water-soluble glutathione and lipid-soluble α-tocopherol in coexisting mixtures of BSA and phosphatidylcholine (PC) liposomes exposed to AEF.

Materials and methods

Materials

Melatonin, glutathione (reduced), α-tocopherol, ascorbic acid, EDTA, 2,4-dinitrophenylhydrazine (DNPH), L-α-PC (XV-E) from egg yolk, trichloroacetic acid (TCA), and bicinchoninic acid protein assay reagents were obtained from Sigma (St. Louis, MO). BSA and ferric chloride were from Bio-Rad Laboratory (Hercules, CA) and Aldrich Chemical (Milwaukee, WI), respectively. A lipid peroxidation (LPO) kit (Calbiochem, La Jolla, CA) was used to measure the quantity of thiobarbituric acid reactive substances, malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). These compounds are end products of LPO chain reactions and are used as an estimate of the degree of LPO. Other chemicals used were of analytical grade and were obtained from commercial suppliers and double distilled water was used for aqueous preparations.

Liposome preparation

Liposome suspension was prepared by dissolving 150 mg PC in 10 ml, a chloroform in a round-bottom flask [31]. Solvent was removed under a nitrogen stream, and the thin film obtained was vortexed for 1 min with 50 ml 0.5 mM phosphate buffer, pH 7.4, and sonicated for 15 min in ice-cold water. The resulting multilamellar liposome of 2 mg PC/ml concentration was used for further experiments.

Oxidation by HO•-generating system

BSA, PC liposome or their mixtures were exposed to the HO•-generating system of AFE composed of 0.1 mM EDTA-Fe^{3+} and 0.5 mM ascorbate in 0.1 M phosphate buffer, pH 7.4, at 37°C for 1 h in 2 ml Eppendorf tubes [32]. For this, 1 mM EDTA-Fe^{3+}, 5 mM ascorbate and 10 mg/ml BSA in 0.2 M phosphate buffer were prepared and used immediately. Briefly 0.1 ml of EDTA-Fe^{3+} solution was added to 0.1 ml of BSA solution, 0.5 ml of PC liposome suspension or 0.6 ml of both after adding 10 µl of acetonitrile. Using 0.19 ml of a 0.2 M phosphate buffer and the same volume of 0.5 mM phosphate buffer, the reaction mixture was adjusted to 0.9 ml. Finally, 0.1 ml ascorbate solution

was added and the mixture was vortexed. The concentrations of BSA and PC in the reaction mixture were 1 mg/ml. After incubation, a 1.1 M mannitol-22 nM EDTA solution was added to give a final concentration of 0.1 M and 2 mM each to stop the reactions.

One percent (v/v) of acetonitrile was used to dissolve and/or disperse antioxidants up to 3 mM. This solvent was also added during glutathione treatment to produce the same mixtures as used with other antioxidants even though acetonitrile is not required to dissolve glutathione. Glutathione was dissolved in 0.2 mM phosphate buffer.

Measurements of protein carbonyl content and protein content

The content of protein carbonyls was determined following the procedure described by Levine et al. [33]. Briefly, to 1 ml samples where the oxidation was stopped with 0.1 ml of mannitol-EDTA solution, 0.2 ml of 10 mM DNPH (dissolved in 2 N HCl) solution was added and the mixture was vortexed followed by incubation at 37°C for 1 h. Ice-cold 0.325 ml of a 50% TCA solution was added to the mixture to give a final concentration of 10%; these were placed on ice for at least 10 min. The pellet obtained after centrifugation at 3,000 g for 10 min was washed three times (followed by sonication between each washing step) with 1 ml ethanol/ethyl acetate (1:1, v/v) to resuspend the protein. The final pellet after the third washing was dried with nitrogen gas and dissolved in 0.7 ml 6 M guanidine-HCl solution, pH 2.0, by vortexing and incubation at 37°C for 15 min. After centrifugation at 13,200 g for 10 min, the absorbance of the supernatant was measured spectrophotometrically at 375 nm to quantify protein carbonyl, and its concentration was expressed as nmol carbonyl groups/mg protein using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ for DNPH derivatives [34]. Guanidine-HCl solution was used as a blank.

Protein content was determined using the bicinchoninic acid protein assay [35]. After the mixture of 5 µl protein solution and 1 ml reagent was incubated at 60°C for 30 min, the absorbance at 562 nm was measured to provide the protein concentration using BSA as standard.

Measurement of amino acid modification

Tryptophan oxidation was monitored by loss of protein fluorescence peaks at 280 nm excitation and 340–350 nm emission in comparison with (untreated and oxidized) free tryptophan. Bityrosine production was assessed at 325 nm excitation and 410–420 nm emission in comparison with authentic bityrosine and bityramine [18].

Determination of LPO

Two hundred microliters of the oxidized samples were mixed with 650 µl of methanol:acetonitrile (1:3) solution containing N-methyl-2-phenylindole followed by vortexing. After adding 150 µl of concentrated methanesulfonic acid, incubation was carried out at 45°C for 40 min. The concentration of MDA + 4-HDA was calculated using the absorbance at 586 nm and 1,1,3,3-tetramethoxypropane as standard; concentrations are expressed as mole per liter [36].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (10%) was performed by the method of Laemmli [37], followed by staining with Coomassie blue. Ten micrograms of BSA was applied per lane.

Statistical analyses

Data were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls t-test. The significance level was accepted at $P<0.05$. All data given are the means of duplicates or triplicates from single experiments, representative of at least two separate experiments. The efficacy of antioxidants to inhibit the oxidation of protein and lipid induced by the AFE system was calculated as follows:

$$\% \text{ Inhibition} = \frac{\Delta\text{AFE control} - \Delta\text{AFE antioxidant}}{\Delta\text{AFE control}} \times 100$$

ΔAFE control refers to the increase of carbonyl or LPO content in the AFE system in the absence of antioxidant; ΔAFE antioxidant refers to the increase of carbonyl or LPO content in the AFE system in the presence of antioxidant.

Results

The increase in the carbonyl content in BSA exposed to AFE system was inhibited by the addition of melatonin in a concentration-dependent manner (Fig. 1) and inhibition reached a maximum of 68% at 3 mM concentration. Acetonitrile, which was used to dissolve the melatonin, interferes with the carbonyl assay much less than other solvents such as ethanol and methanol which are usually used to aid melatonin's solubility in the water phase. Acetonitrile was used to dissolve melatonin up to a concentration of 3 mM in the water phase. One percent (v/v) acetonitrile was selected in which to dissolve melatonin since it was highly effective and minimally interfered with the carbonyl assay (data not shown), i.e., the acetonitrile was the maximal amount to dissolve

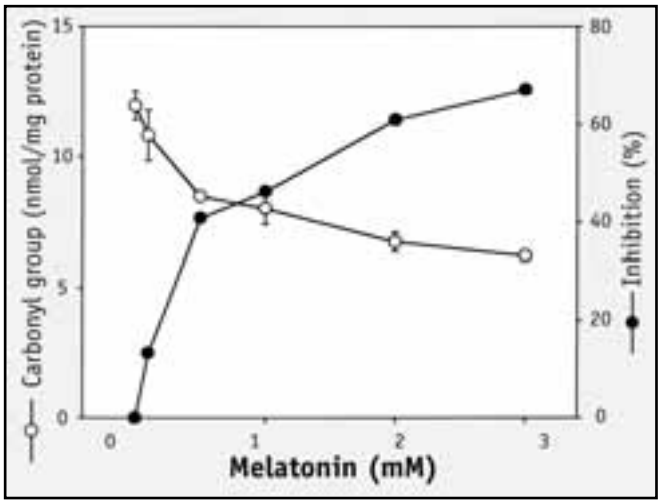

Fig. 1. Inhibition of carbonyl formation in BSA by melatonin. BSA (1 mg/ml) was exposed to ascorbate-Fe^{3+}-EDTA (AFE) consisting of 0.1 mM EDTA-Fe^{3+} and 0.5 mM ascorbate in 0.1 M phosphate buffer, pH 7.4, at 37°C for 1 h and carbonyl formation was measured by treating with 10 mM 2, 4-dinitrophenylhydrazine (DNPH). 1% acetonitrile (v/v) was used to dissolve melatonin up to 3 mM in the reaction mixture. Inhibition (%) is the ratio of the carbonyl levels reduced by melatonin to levels increased by AFE without melatonin. Data represent the means ± SE of two measurements in triplicate.

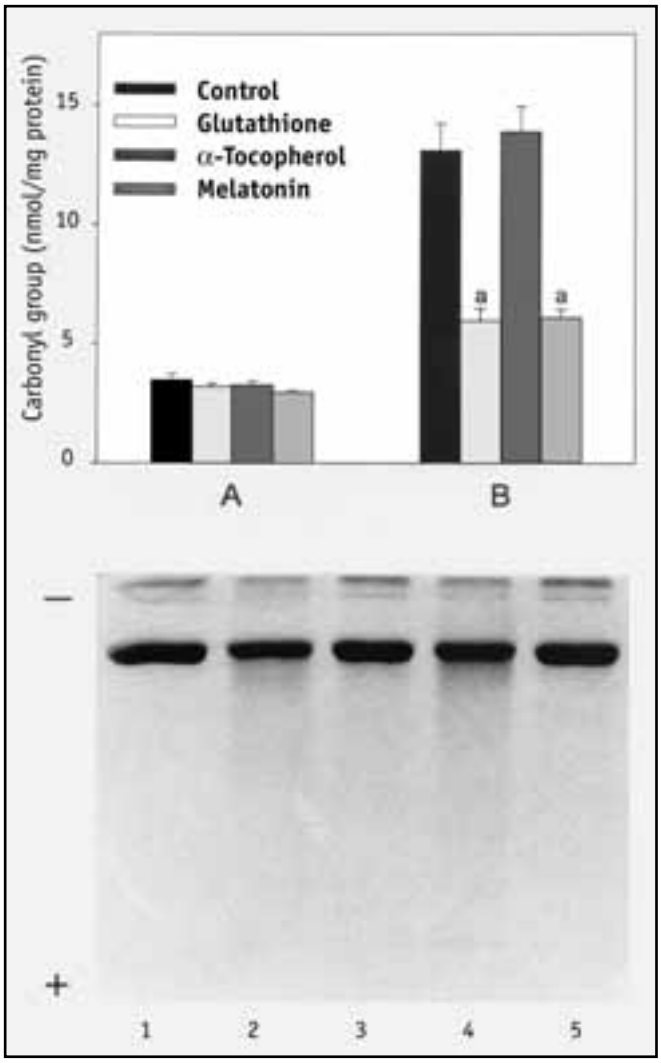

Fig. 2. Effect of antioxidants on carbonyl formation (top) and SDS-PAGE profile (bottom) in BSA exposed to AFE. 3 mM concentrations of antioxidants were used; the other conditions were the same as in Fig. 1. A and B in top figure indicates BSAs not exposed and BSAs exposed to AFE, respectively; the control samples lacked antioxidants. Data represent the means ± SE of four measurements in duplicate and (a) indicates significantly different at $P<0.001$ from control exposed to AFE without antioxidant. Bottom figure shows the electrophoresis of BSAs (10 µg per lane of 10% SDS-PAGE) with different treatments: lane 1, BSA standard; lane 2, BSA exposed to AFE; lane 3, lane 2 + glutathione (3 mM); lane 4, lane 2 + α-tocopherol (3 mM); lane 5, lane 2 + melatonin (3 mM).

as much melatonin as possible without significantly influencing the carbonyl content by the addition of only acetonitrile to the reaction mixture. Without acetonitrile, 0.3 mM melatonin could be dissolved in the water phase and it had an inhibitory effect on carbonyl formation of about 14% (data not shown).

Comparison of the capacity of antioxidants to inhibit carbonyl formation showed that melatonin (70 ± 2%) and glutathione (73 ± 3%) were equally effective while α-tocopherol (9 ± 3%) was ineffective (Fig. 2, top). Analysis of BSA fragmentation by SDS-PAGE revealed similar results (Fig. 2, bottom); thus, melatonin and glutathione prevented fragmentation whereas α-tocopherol did not.

Melatonin (85 ± 7%) reduced LPO of PC liposomes induced by AFE more effectively than did α-tocopherol (51 ± 4%) (Fig. 3). Glutathione (29 ± 5%) had only a weak effect in protecting against lipid peroxidation.

In PC liposome containing BSA, 3 mM melatonin prevented effectively both protein (carbonyl: 62 ± 6%, SE) and lipid (LPO: 83 ± 9%, SE) damage while glutathione (carbonyl: 76 ± 8%, LPO: 16 ± 4%) was mainly effective in protecting protein while tocopherol (carbonyl: 2 ± 6%, LPO: 54 ± 11%) was exclusively a lipid protector (Fig. 4, top and 5). The fragmentation of BSA was inhibited similarly by melatonin and glutathione but not by α-tocopherol (Fig. 4, bottom).

It was not possible to measure the effect of melatonin in altering bityrosine formation or the loss of tryptophan residues in oxidized BSA since melatonin strongly interfered with the measurement of fluorescence.

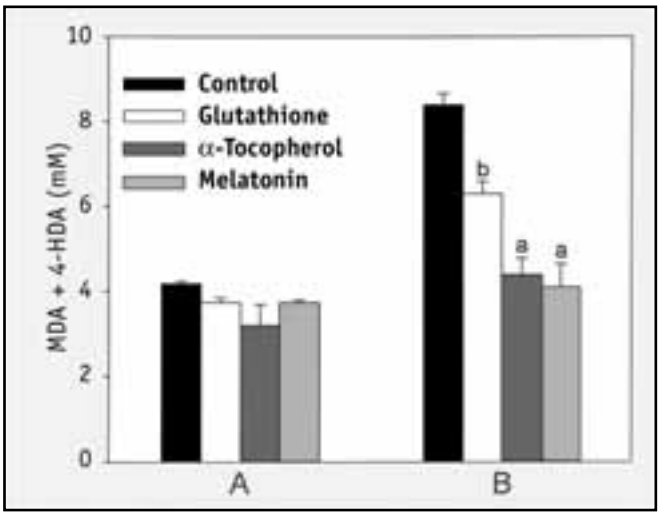

Fig 3. Inhibition of lipid peroxidation (LPO) by antioxidants in phosphatidylcholine (PC) liposome. LPO in PC liposome (1 mg/ml) was induced by AFE as in Fig. 1. The content of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) was used as an index of LPO and the capacity of each antioxidant at 3 mM was compared. A, B and Control represent the same as in Fig. 2. (a) and (b) indicate significantly different at $P<0.001$ and $P<0.005$ from control exposed to AFE without antioxidant, respectively.

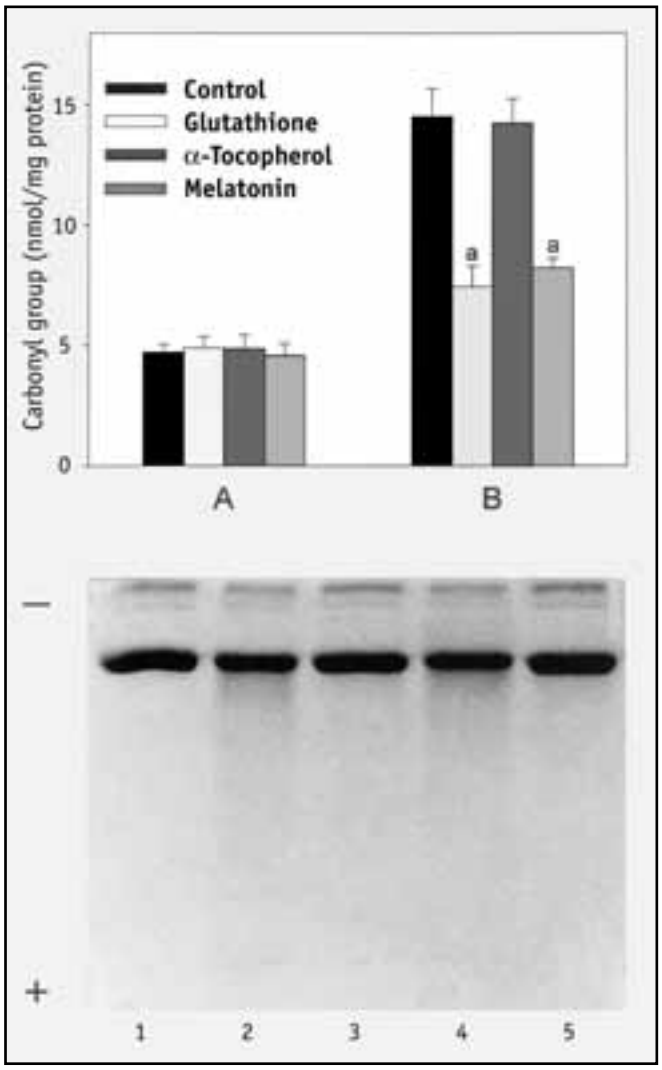

Fig. 4. Effect of antioxidants on carbonyl formation (top) and SDS-PAGE profile (bottom) in BSA coexisting with PC liposome in their mixture. PC liposome containing BSA of 1 mg/ml was exposed to AFE as in Fig. 1. The conditions for comparing the efficacy of the antioxidants and the meaning of symbols are the same as in Fig. 2.

Discussion

The HO• is known to be a major initiator of damage to protein from studies using ionizing radiation [18] and metal-catalyzed oxidation (MCO) [22]. It is generally accepted that HO•-dependent abstraction of a α-hydrogen atom from an amino acid residue initiates the oxidative attack of the polypeptide backbone to form a carbon-centered radical [18]. The major difference in damage between that induced by radiation and that which occurs as a consequence of MCO is the degree of modification of amino acids; thus, ionizing radiation modifies almost all amino acids whereas MCO alters fewer amino acid residues. AFE, as used in the present studies, induces HO• in this system through the well-known Fenton reaction. Miura et al. [38] has measured the production of HO• using the hydroxylation of salicylate. The presence of EDTA in the ascorbate system is known to increase protein carbonyl formation [22, 39] though the role of EDTA is still uncertain. Stadtman [19] presumed that some proteins such as BSA lack a high affinity site for metal ions but can bind EDTA-metal ion complexes; thus, EDTA serves as a carrier of the metal ion to protein and the interaction of the EDTA-metal ion-protein complex with hydrogen peroxide (H_2O_2) leads to an increased site-specific generation of $HO^{\bullet}$ [39]. These HO• may damage BSA via several means which introduce carbonyl onto the protein [18]. The carbonyl content of native BSA (3.5 nmol/mg protein) is usually increased about 3.7 fold in the AFE system.

Carbonyl formation in the AFE system was inhibited effectively by both melatonin and glutathione (Fig. 2), which are known HO• scavengers [7, 40, 41]. Stadtman [19] reported that it is difficult to inhibit protein damage by MCO using scavengers because the production of HO• occurs only at the sites where iron is bound. Davies et al. [18] and Miura et al. [38], however, reported HO• scavengers including mannitol, ethanol and uric acid prevent protein damage in this system. Because carbonyls are also formed by the reaction of protein with products of LPO such as MDA and 4-hydoxynonenals [42, 43], Blakeman et al. [44] surmised that an antioxidant's efficacy in inhibiting carbonyl formation may be due to its ability

to prevent the peroxidation of lipid contaminants in BSA not directly on carbonyl formation. Even though the BSA used in the current study was a highly purified standard, the level of lipid peroxidation products present was investigated with and without AFE addition. The outcomes of these studies did not differ (data not shown), indicating that lipid peroxidation was not involved. The thiol groups of glutathione also are known to react with HO•; this could account for the ability of glutathione to reduce carbonyl formation. The studies of Tan et al. [7] indicate that melatonin is a better HO• scavenger than is glutathione, although in the present study their abilities to inhibit protein oxidation in aqueous solution were similar (Fig. 2). These seemingly different outcomes may be a consequence of the different measurement systems used to estimate the antioxidant efficacies of these molecules. Tocopherol was ineffective in reducing protein carbonyl formation, presumably, due to its insolubility in aqueous media where HO• were formed. Yan et al. [45] reported similar results using different oxidants and BSA; thus, glutathione was an effective inhibitor of carbonyl formation while α-tocopherol was not.

Protein fragmentation of BSA also occurred in the present study (Fig. 2, bottom). The fate of damaged proteins is determined by the availability of O_2 and $O_2^{\bullet -}$ or its protonated form (HOO•). In the presence of O_2, proteins are mainly fragmented while they aggregate in the absence of O_2 [18,19]. Protein fragmentation was a predominant feature in our AFE system since it contained O_2. This fragmentation was inhibited both by melatonin and glutathione, but not by tocopherol; again, this was presumably due to the lack of solubility of the vitamin in aqueous media.

Acetonitrile, which was used for aiding melatonin's dissolution in water, did not interfere with the measurements. Melatonin's ability to protect against carbonyl formation when we attempted to dissolve the indole in acetonitrile-free water phase was weak (3% and 14% inhibition at 0.1 and 0.3 mM of melatonin, respectively). This was likely due to the limited solubility of melatonin in the water phase.

Melatonin's potential to protect against lipid peroxidation was compared with both vitamin E and glutathione in a lipid environment. Melatonin had a greater inhibitory capacity against LPO than did tocopherol; vitamin E is known to be a very effective lipid antioxidant [46, 47] because of its ability to block the chain reaction involving ROO•. Like tocopherol, melatonin reportedly scavenges ROO• although there is some disagreement as to its effectiveness in this regard. Pieri and co-workers [9] reported that melatonin is a better ROO• scavenger than is tocopherol, but other studies [10–12] indicate that melatonin is less efficient than tocopherol in the direct detoxification of the ROO•. It would seem the most likely explanation for melatonin's ability to reduce LPO in the current studies relates primarily to its ability to scavenge the initiating radicals and secondarily to its ability to detoxify ROO• [48]. Thus, melatonin presumably prevented the initial step in the process of LPO by scavenging highly toxic radicals and interrupted the process by detoxifying ROO•. Melatonin's ability to reduce LPO has been amply demonstrated [16, 30]. Glutathione's slight inhibitory effect in LPO even though it is not dissolved in the lipid phase may come from its ability to scavenge HO• in the water phase.

Melatonin is highly soluble in lipid [27] and much less so in water [28]. Livrea et al. [12] reported that 35% of the melatonin from an unilamellar liposome diffuses into the aqueous phase, both in soybean and dimirystoyl PC liposomes. This unusual solubility of melatonin may be beneficial for its functions as an antioxidant since radicals are produced in all parts of the cell and migrate only a few Ångstroms from their site of origin due to their high reactivity; thus, for a direct free radical scavenger to neutralize a radical, it must essentially be at the site where the radical is generated [48]. To investigate the potential of melatonin in radical scavenging and biomolecular protection against radicals produced in water phase, we used PC liposomes containing BSA, that is, as a mixture of biomolecules in lipid and water phases. In this system, melatonin prevented both protein and lipid damage efficiently while glutathione and tocopherol were mainly effective in preventing damage to only protein (glutathione) or lipid (tocopherol). The HO•-scavenging ability of melatonin in the water phase presumably explains its efficacy in inhibiting BSA oxidation and some LPO initiation. Conversely, the HO• and to a lesser extend ROO•-scavenging activity of melatonin in the lipid phase possibly curtailed LPO.

Unlike melatonin and glutathione, tocopherol significantly inhibited LPO in the control samples without AFE (Fig. 5, A). This inhibition is likely due to the ability of vitamin E to scavenge ROO• formed during LPO initiated by some impurities in PC. Melatonin was less effective than tocopherol in this situation probably related to its weaker scavenging activity of the ROO• [10,16].

Although it is generally accepted that HO• are potent initiators of lipid peroxidation, there are some reports that HO• are not as efficient as hydroperoxide (HOO•) and ROO• in the initiation of LPO [49]. If this is the case, the inability of HO• to initiate LPO outside the liposome and considering the limited ability of melatonin to scavenge the ROO•, another possibility by which melatonin so efficiently protects against LPO might have to be considered.

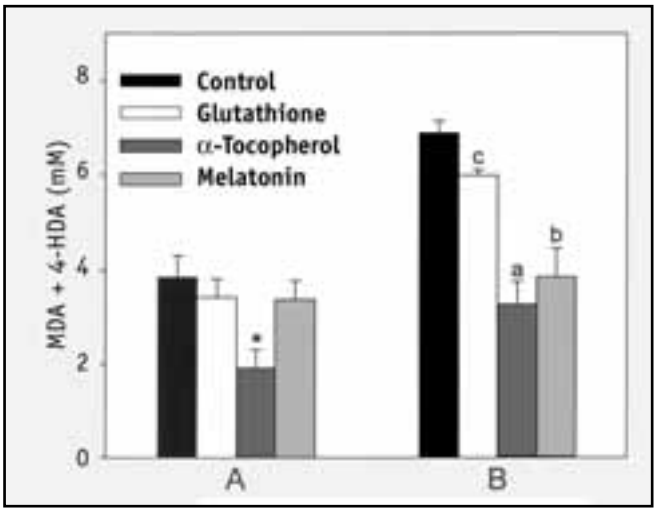

Fig. 5. Inhibition of LPO by antioxidants in PC liposome containing BSA. LPO levels in the samples tested in Fig. 4 were measured. The conditions for the experiment and the meaning of symbols are the same as in Fig. 3. (a), (b) and (c) indicate significantly different at P<0.001, P<0.005 and P<0.05 from control exposed to AFE without antioxidant, respectively. * indicates significantly different at P<0.05 from control without AFE and antioxidant.

One possibility might be that melatonin scavenges HO• in lipid phase since sub-μM concentrations of iron are known to drive the Fenton reaction [49, 50] in that phase. However, this seems unlikely since HO• production by AFE system in lipid phase may be impossible because ascorbate as a reductant does not enter the lipid phase [51].

Finally, these results with melatonin may be explained by what is known about melatonin. Firstly, melatonin is an efficient HO• scavenger [7, 8, 40, 41] and it may also scavenge to some degree the ROO• [9–12]. Secondly, melatonin can act both in the aqueous and lipid phases. The ability of melatonin to scavenge HO• prevents protein damage and the initiation of LPO in water phase and its possible ROO•-scavenging activity in the lipid phase slows the propagation of LPO. In reference to nuclear DNA, melatonin has also been shown to reduce damage to this molecule which is a consequence of the •OH [52, 53]. Further study will be necessary, however, to totally characterize melatonin's mechanisms of action in these phases.

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