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Oxidative Damage to Catalase Induced by Peroxyl Radicals: Functional Protection by Melatonin and Other Antioxidants

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Thermal decomposition by the azo initiator 2,2′ azobis-(2-amidinopropane) dihydrochloride (AAPH) has been widely used as a water-soluble source of free radical initiators capable of inducing lipid peroxidation and protein damage. Here, in a lipid-free system, AAPH alone (40 mM) rapidly induced protein modification and inactivation of the enzyme catalase (EC 1.11.1.6). Using SDS-PAGE, it was shown that protein band intensity is dramatically reduced after 4 h of incubation with AAPH, leading to protein aggregation. Several antioxidants including melatonin, glutathione (GSH) and trolox prevented catalase modification when used at a 250 μM concentration whereas ascorbate was only effective at 1 mM concentration. All the antioxidants tested reduced carbonyl formation although melatonin was the most effective in this regard. Enzyme inactivation caused by AAPH was also significantly reduced by the antioxidants and again melatonin was more efficient than the other antioxidants used in this study. Results shown here demonstrate that alkyl peroxyl radicals inactivate catalase and reduce the effectiveness of cells to defend against free radical damage; the damage to catalase can be prevented by antioxidants, especially melatonin.

Keywords: Melatonin; Enzyme modification; Free radicals; AAPH; Peroxyl radicals; Catalase

Abbreviations: AAPH, 2,2′-Azobis(2-amidinopropane) hydrochloride; MDA, Malondialdehyde; HNE, 4-hydroxy-2-nonenal; ROS, Reactive Oxygen Species; DNPH, 2,4-Dinitrophenylhydrazine; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

INTRODUCTION

Melatonin, or N-acetyl-5-methoxytryptamine, is a secretory product of the pineal gland of mammals and in this organ most vertebrates produce increased amounts of the indole during the dark phase of the circadian cycle.[1] However, its production is not confined exclusively to this gland and other organs and tissues have been reported to produce it as well.[2,3] Melatonin is also synthesized in invertebrates and in other organisms including dinoflagellates, algae and bacteria.[4] The presence of melatonin in such a variety of organisms suggests that this substance is phylogenetically highly conserved and may play an important role in the function of organisms.

Until recently, much of the research in experimental animals focused on the interactions of melatonin with the neuroendocrine–reproductive system, although its actions are widespread. Thus, melatonin has been classically associated with circadian and circanual rhythm regulation, and with adjustments of the physiology of animals to seasonal environmental changes.[1] Besides its function in the control of annual fluctuations in reproductive competence due to seasonally changing photoperiods, during the last decade melatonin has been shown to have antioxidant properties and to protect against a number of toxic species.[5,6] The scavenging of several oxygen-based reactants by melatonin protects against oxidative stress-related processes in experimental models of aging and neurodegenerative disorders.[7] Numerous studies have reported on melatonin's protection against lipid peroxidation and DNA damage induced by reactive oxygen species (ROS) both *in vivo*

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and *in vitro*,[8,9] but there is a paucity of experimental data on melatonin's efficacy in reducing oxidative protein damage.

ROS are generated *in vivo* during the partial reduction of oxygen during mitochondrial respiration or when organisms are exposed to toxic environmental agents. The resulting reactants, some of which are free radicals, can damage biomolecules such as DNA, lipids and proteins, resulting in compromised cellular functions. As a consequence, ROS are believed to be involved in the etiology of a variety of pathologies, some of which are related to aging, e.g. atherosclerosis, arthritis, cataractogenesis, cancer and several neurodegenerative diseases.[10,11] Thus, oxidative modification of macromolecules due to ROS is receiving increased attention.[12,13] Oxidative modification of proteins by ROS increase with age and these damaged products have been implicated in the etiology and/or progression of several disorders and diseases.[14,15] In recent years, several groups have focused their research to studies on structural and enzyme protein modification by free radicals.[16,17]

Exposure of proteins to either the $^{\bullet}OH$, $O_2^{\bullet -}$ or both leads to gross structural modifications that can trigger spontaneous protein fragmentation and/or cross-linking. A wide variety of reactions between ROS and amino acid chains occurs and all amino acids in proteins are susceptible to modification by $^{\bullet}OH$ or by a combination of $^{\bullet}OH$ and $O_2^{\bullet -}$.[18] In addition to fragmentation, the oxidation of lysine, arginine, proline and threonine residues may also yield carbonyl derivatives. The presence of carbonyl groups, has therefore, been used as a maker of ROS-mediated protein oxidation.

Oxidative modification of proteins is also caused by aldehydes produced during lipid peroxidation. In this process, end-products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) as well as products resulting from polyunsaturated fatty acid damage cause protein breakdown.[13] Experimentally, alkyl-peroxyl radicals initiated by thermal decomposition of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) are responsible for the oxidation of proteins.[19,20] The oxidative modification and, consequently, inactivation of antioxidant enzymes is particularly interesting, as these enzymes represent the major antioxidant system in many eukaryotic cells.

The aim of the present study was to evaluate the pro-oxidant action of alkyl-peroxyl radicals generated by the azo initiator AAPH and their potential for damaging the enzyme catalase (EC 1.11.1.6). In addition, we examined the possible protective role of melatonin and other antioxidants in this model.

MATERIALS AND METHODS

Materials

Catalase, glutathione, ascorbate, phenol-red and horse radish peroxidase were purchased from Sigma Chemical Company (St Louis, MO, USA). 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Fluka (Neu-Ulm, Switzerland). AAPH was obtained from Wako Chemicals USA Inc. (Richmond, VA, USA). Trolox was purchased from Aldrich (Milwaukee, WI, USA). Ultra pure grade melatonin was a kind gift from Helsinn Chemical Company (Biasca, Switzerland).

Alkyl-peroxyl Radical Mediated Protein Damage

Catalase (Sigma Co, # C-9322) was dissolved at a final concentration of 0.5 mg/ml (1.195 units/ml) in 150 mM air-saturated phosphate buffer, pH = 7.3. AAPH was prepared as a 700 mM stock solution in water and heated for 5 min at 37°C before addition to the samples. In these experiments, the same amount of water was added to the control groups. Reactions were performed in opened tubes placed in a shaking water bath at 37°C. Melatonin and trolox were dissolved in 100% ethanol and added from a 500 mM stock solution to minimize the final concentration of ethanol. Vehicle (ethanol, 0.2% v/v) was always included in the AAPH treated group. Glutathione and ascorbate were directly dissolved in 150 mM phosphate buffer.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine and quantify protein damage, after treatment with the different reagents, protein samples were subjected to SDS-PAGE according to Laemmli's method[21] using the Mini-Protean® 3 system (Bio-Rad Laboratories, CA, USA). Samples were mixed with a four times concentrated loading buffer (40% Glycerol, 8% SDS, 0.25 mM Tris-HCl pH = 6.8, 20% β-mercaptoethanol, 0.01% bromophenol blue) and heated at 100°C for 2 min. Ten micrograms of protein sample were loaded in a 12.5% polyacrylamide gel and electrophoresed. After running 1 h, gels were stained with 0.15% Coomasie Brilliant Blue R-250. This dye forms complexes with most proteins, through the combination with NH_3^+ groups so the uptake of dye by proteins is approximately proportional to the amount of protein, following the Beer–Lambert Law.[22] Following staining, gels were washed extensively with ethanol:acetic acid:water (40:10:50) and dried in a gel dryer (Bio-Rad) for 45 min at 80°C. At least four gels for each experiment were prepared.

Measurement of Band Intensity and Graph Representation

To determine the amount of protein damage, band intensity was measured using the Scion Image™ Beta 4.02 for Windows™ analysis software, downloaded at the web site address. E-mail: http://www.scioncorp.com, using the gelplot2 macro for the measurements, with a previous image calibration. The optical density of each band was estimated and standardized with respect to the control group. Results show the average of at least four different measurements from independent experiments.

Determination of Carbonyl Content

The carbonyl content of proteins was quantified by the reaction with DNPH, using the method described by Levine *et al.*[23]. After stopping the reaction by the addition of 0.2 ml of 1 M mannitol solution, 2 ml samples containing 1 mg total protein (four samples per group) were mixed with 0.5 ml of 10 mM DNPH and reacted for 1 h at 37°C to give a final concentration of 2 mM of DNPH. Then, 0.625 ml of 50% ice-cold trichloroacetic acid was added. After 10 min of incubation on ice, samples were centrifuged at 13 000*g* 5 min. Pellets containing the proteins were washed three times with ethanol/ethyl acetate (1:1 v/v). Finally the pellets were resuspended in 1 ml of 6 M guanidine in 2N HCl pH = 2.0 by vortexing, and incubated at 37°C for 15 min. After this time, samples were clarified by centrifugation at 13 000*g* for 5 min and the absorbance of supernatants was read at 375 nm in a Beckman DU 530 Spectophotomer. Protein carbonyl content, expressed in nmol per mg of protein, were estimated by using the molar absorption coefficient (ϵ) of 22 000/M/cm for DNPH derivatives. According to the original method, purified proteins do not require reagent blanks to be run in parallel, as it happens with a mixture of proteins.[23] Thus, protein samples untreated with DNPH and resuspended in guanidine-HCl solution were used as a blank. Data were standardized by measuring the protein content using the Bradford method and the Bio-Rad kit.

Measurement of Catalase Activity

To determine catalase activity, an indirect method based on the measurement of H_2O_2 concentration was used as described.[24] Briefly, after incubation of catalase (0.5 mg/ml; 1.195 U/ml) with or without AAPH, in the presence or absence of the antioxidants was performed. One aliquot of these samples was incubated with 6 mM H_2O_2 in 150 mM phosphate buffer pH = 7.3 for 30 min at 37°C. After this interval, 10 µl were added to 1 ml reaction solution (10 mM potassium phosphate pH = 7.0, 140 mM NaCl, 0.1 mg/ml phenol red, 8.5 U/ml horseradish peroxidase, HRP) and incubated for 5 min at room temperature. Then, 10 µl of 1N NaOH were added to raise the pH to 12.5 and the absorbance at 610 nm was measured in a Spectronic 601 Spectrophotometer. A solution of 60 µM H_2O_2 without and with catalase was used as positive controls to calibrate the reaction and estimate 100% of catalase activity in control samples. Results are expressed as nmol H_2O_2 consumed per mg protein per min, or as % of control value. All the experiments were repeated at least three times.

Statistical Analysis

Data are presented as means ± SEM of at least three different experiments using four samples per group. One-way ANOVA was performed to compare different groups, followed by a Student–Newman Keuls *t* test. Statistical significance was considered when $P < 0.05$.

RESULTS

AAPH Induces Catalase Modification

Figure 1 (A–D) represents the results obtained after treatment of catalase with 40 mM of AAPH for the intervals indicated. Results represent the PAGE analysis of the protein (A), density of protein band (B), carbonyl content (C) and the effect on enzyme activity (D). As shown in the gel (Fig. 1A) and in the corresponding graph (Fig. 1B), there is an obvious reduction of the protein band (arrow, ←) even after 2 h of treatment when compared to control (C). Alkyl peroxyl radicals generated by thermal decomposition of AAPH induced protein aggregation (arrowhead, ◄) during short-term incubation and complete degradation after 8 h. Figure 1C shows the increase in the carbonyl content of catalase after AAPH treatment. The increase is very rapid during the first 8 h, and reaches a plateau after 24 h of incubation with the azo compound (Fig. 1C). Finally, enzyme activity declines quickly and after 2 h of incubation, catalase activity is roughly 50% that of control values (Fig. 1D).

Antioxidants Prevent Catalase Structural Damage Induced by AAPH

The efficacies of melatonin, GSH, ascorbate and Trolox in reducing protein damage induced by AAPH is shown in Fig. 2 (A–D). Except for ascorbate, antioxidants tested in the present study were efficient protectors at concentrations greater than 500 µM. For ascorbate only, the highest concentration used (1 mM) was found to reduce

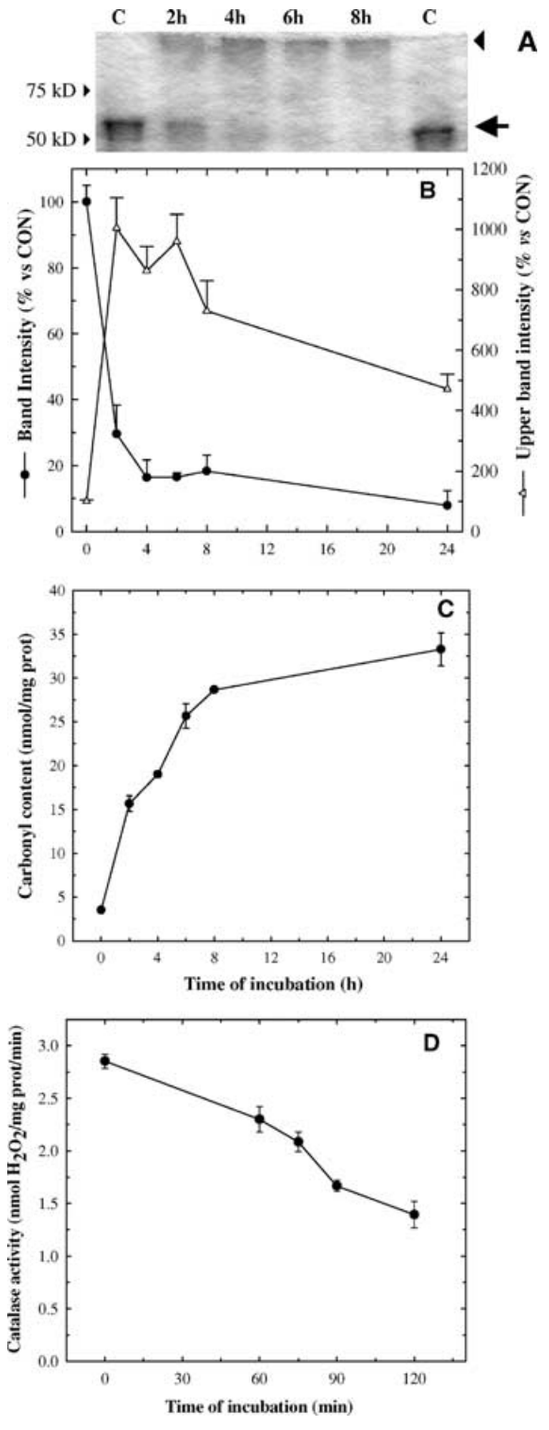

FIGURE 1 Time course study on the effect of AAPH on catalase protein. Catalase (0.5 mg/ml) was left untreated (C) or incubated in the presence of AAPH (40 mM) for the times indicated at 37°C. PAGE analysis was performed with 10 μg of protein per lane and the gels were stained with Coomasie blue; molecular weight markers are shown on the left (small arrowheads); the corresponding catalase band (arrow ←, right) and protein aggregation (large arrowhead ◄, right) are shown (A). Densitometric analysis of protein bands shown in (A), from three different experiments (B). Carbonyl content (C) and enzyme activity (D) were estimated from parallel experiments as described in Materials and Methods section using at least four samples per group and results from a representative experiment are shown.

damage to catalase with statistical significance, although a tendency to a protective effect is also observed when 0.5 mM was used (Fig. 2C). Melatonin was more efficient than any other antioxidant and concentrations as low as 100 μM reduced both protein aggregation and degradation (Fig. 2A). Likewise, trolox was also efficient in preventing alkylperoxyl radical-mediated damage of catalase (Fig. 2C), but according to the densitometric studies, it never restored the protein band intensity to the control level. Finally, the other physiological antioxidant, GSH, although efficient in preventing protein damage at the highest concentrations of >500 μM, failed to preserve the protein band when used at lower concentrations; the protective effect of GSH was either total or it was ineffective (Fig. 2B). For all antioxidants, the protective effect in terms of protein aggregation (data not shown) was equivalent to the changes obtained with the main protein band intensity, indicating that the initial damage generated by AAPH is a result of protein cross-linking.

Antioxidants Prevent Carbonyl Content Increase Induced by AAPH

In addition to the results of the PAGE analysis, Fig. 3 shows that all the antioxidants efficiently prevented protein carbonyl formation induced by AAPH. Melatonin almost completely returned basal carbonyl content to the control levels (Fig. 3A), whereas the other antioxidants only partially prevented carbonyl accumulation. None of the antioxidants tested reduced carbonyl formation when used at a 100 μM concentration while trolox and ascorbate did not lower the increase in carbonyl even at a concentration of 250 μM (Fig. 3C and D, respectively).

Melatonin's effect on carbonyl group formation was studied over time and the results are summarized in Fig. 4. Melatonin was found to inhibit carbonyl formation when catalase was incubated in the presence of the indole for 24 h, showing that melatonin's effect persists over time; the maximal preventive effect of melatonin was seen at 6–12 h.

Antioxidants Prevent Catalase Inactivation Induced by AAPH

As shown in Fig. 1D, AAPH induced a rapid enzyme inactivation when catalase was incubated in the presence of the azo initiator for 2 h. In this model, the protective effect of antioxidants on catalase inactivation was determined by measuring the activity of the enzyme. Melatonin was again the most effective in preventing catalase inactivation (Fig. 5), although all antioxidants tested restored enzyme activity when used at higher concentrations (>250 μM). Melatonin (Fig. 5A) was effective at doses as low as

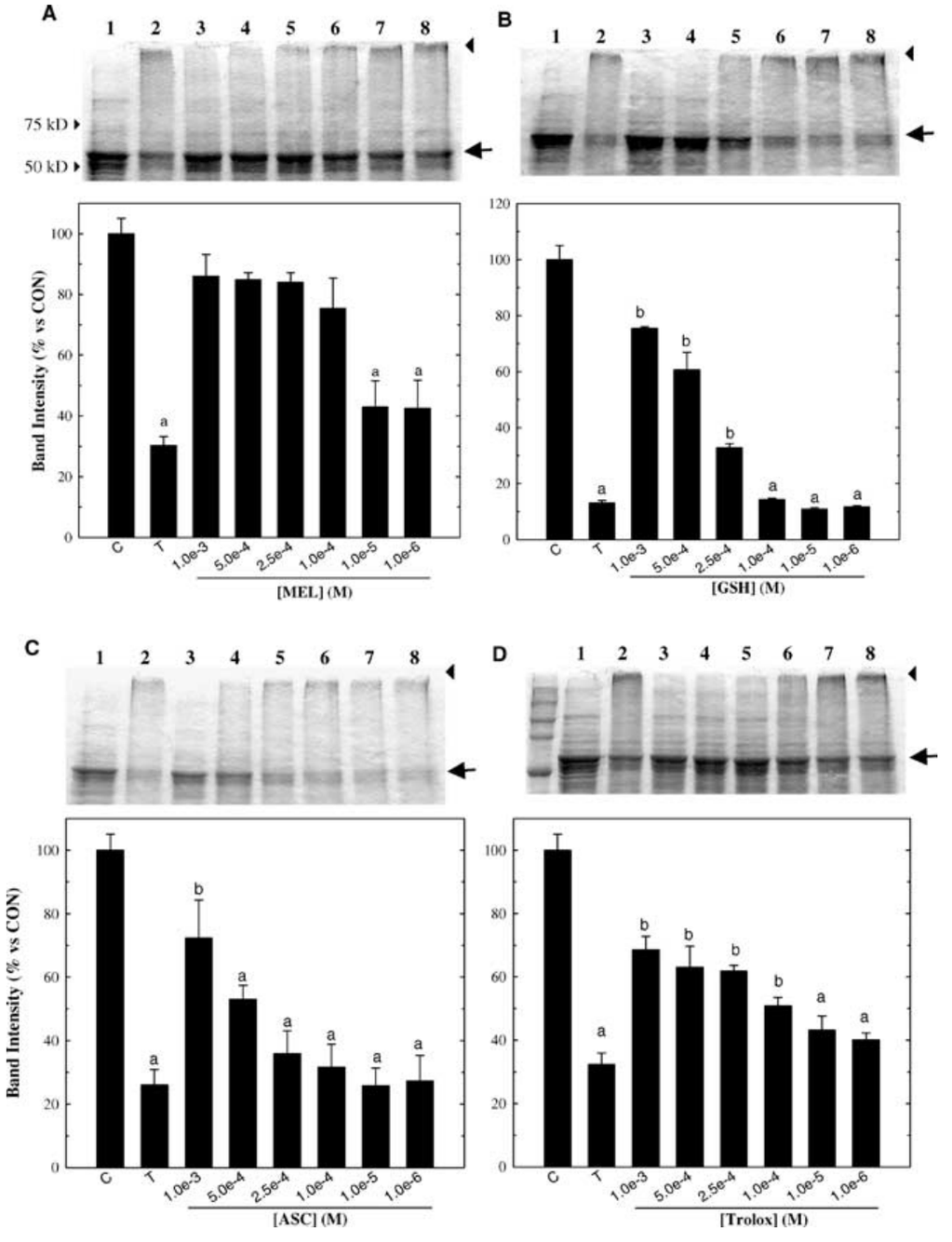

FIGURE 2 Protective effects of different antioxidants on catalase on AAPH-induced damage. Catalase (0.5 mg/ml) was incubated in the absence (C, control) or presence of AAPH (T) with the addition of the antioxidants melatonin (A), GSH (B), ascorbate (C) or trolox (D) for 4 h at 37°C at the concentrations indicated. Electrophoretic patterns from PAGE and graphs showing the densitometric analysis of protein bands are shown. Ten micrograms of protein were loaded in each lane. Results represent the mean ± SEM from at least four independent experiments. Arrow (←), catalase protein band; large arrowhead (◄, right), protein cross-linking. (a) $P < 0.05$ vs. remain of groups; (b) $P < 0.05$ vs. CON group.

100 μM. GSH was only completely effective at a concentration of 1 mM (Fig. 5B), although it had a partial effect at 250 μM. Finally, ascorbate and trolox were more effective than glutathione in preventing the reduction in catalase activity; both antioxidants at 1 mM and 250 μM concentrations totally prevented enzyme inactivation (Fig. 5C and D, respectively). It is noteworthy that both GSH and trolox, at the lowest concentrations, did not only not protect but unexpectedly enhanced the decrease of enzyme activity induced by AAPH (Fig. 5B and D, respectively).

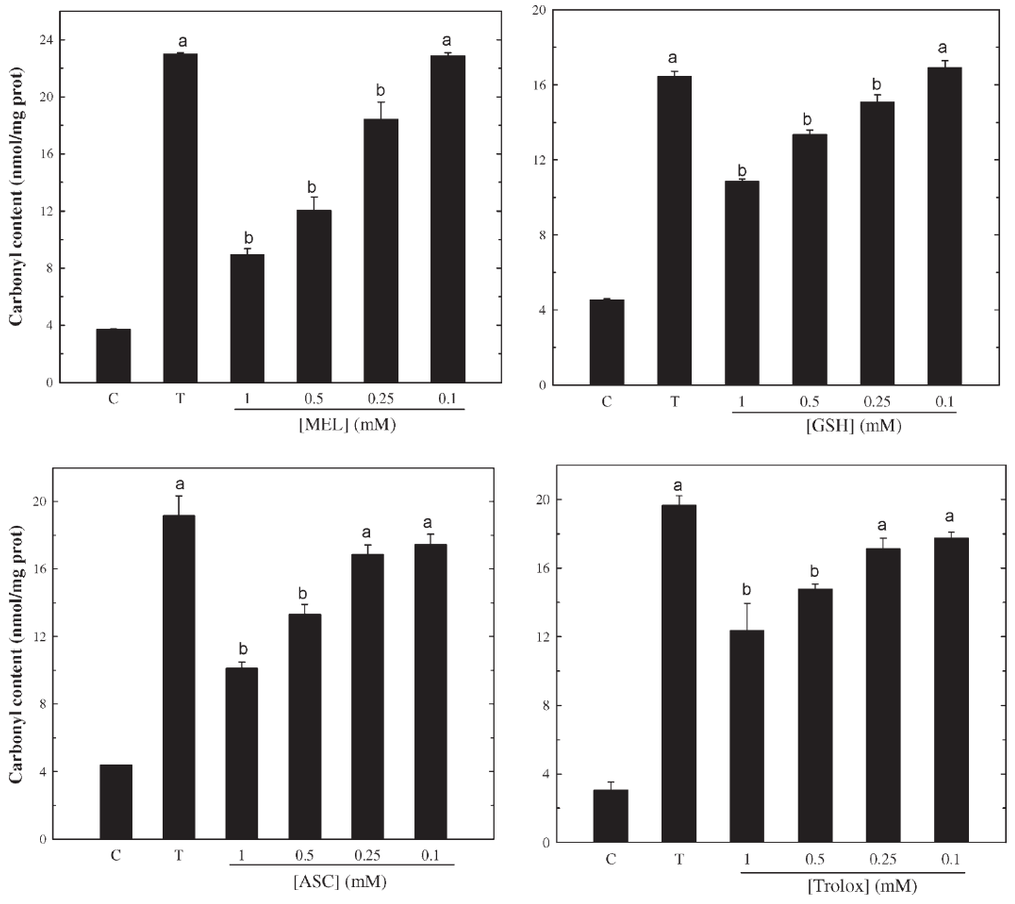

FIGURE 3 Effects of antioxidants on the carbonyl content of catalase. Catalase (0.5 mg/ml) was incubated in the absence (C, control) or in the presence (T) of 40 mM AAPH at 37°C, with the antioxidants melatonin (A), GSH (B), ascorbate (C) or trolox (D) for 4 h, at the concentrations indicated (0.1–1 mM). Carbonyl content was measured as described in the Materials and Methods section. Results displayed correspond to the mean ± SEM of at least three independent experiments. (a) $P < 0.05$ vs. remain of groups; (b) $P < 0.05$ vs. control group (CON).

The effects of these antioxidants on catalase activity are compared in Fig. 6. Melatonin was clearly the most effective in preserving the enzyme activity and preventing the inactivation of catalase by AAPH. The IC_{50} values for the protective effects on the catalase activity for melatonin, GSH, ascorbate and trolox were 30 μM, 300 μM, 100 μM and 80 μM, respectively.

DISCUSSION

In the present work, we report for the first time that alkyl-peroxyl radicals formed by AAPH induce oxidative damage to catalase, as assayed by staining of PAGE with Coomassie brilliant blue, resulted in the rapid loss of enzyme activity (Fig. 1). Also, we show for the first time that melatonin efficiently prevents protein damage and enzyme inactivation induced by AAPH. Melatonin reduces protein fragmentation and carbonyl content in catalase (Figs 2 and 3, respectively) and, more importantly, preserves the activity of this enzyme to catalyze the reaction with H_2O_2 (Fig. 5). Furthermore, melatonin was one of the best antioxidants tested in the present study in terms of their abilities to scavenge alkyl-peroxyl radicals indicated by the reduction in protein structural damage and in reducing the decline in enzyme activity due to oxidative modification. Melatonin's protective actions are therefore not restricted to DNA and lipids as shown in previous studies,[12,14] but it also protects the protein catalase from oxidative damage.

The antioxidant effects of melatonin against a variety of free radical species in protecting DNA and lipids from oxidative deterioration have been widely investigated *in vitro*, in cell culture and in *in vivo* models, e.g. in ischemia/reperfusion injury and in experimental neurodegenerative conditions.[9,25,26] In contrast, only recently have the protective effects of the indole against oxidative protein modification induced by metal-catalyzed oxidation been investigated in detail.[27–29] Several authors have noted a reduction in carbonyl content and nitrotyrosine

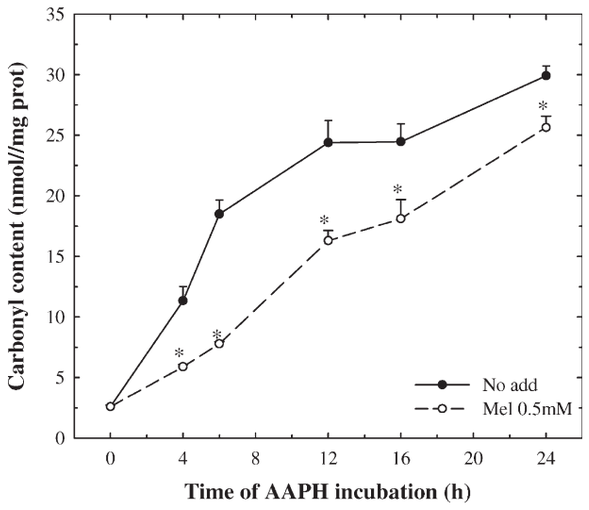

FIGURE 4 Time-course study of melatonin's effect on the carbonyl content of catalase. Catalase (0.5 mg/ml) was untreated or incubated with 40 mM of AAPH for the times indicated in the absence or in the presence of 500 μM of melatonin. Carbonyl group determination was estimated as described (see Materials and Methods section). Results show the mean ± SEM of three independent experiments. $*P < 0.05$ vs. AAPH group without additives.

residues due to melatonin treatment in some *in vivo* models,[30] highlighting the importance of general protection afforded by the indole against free radical damage. While protection of DNA and lipids from radical attack are generally considered important in slowing age-related cellular degeneration, it is also recognized that reducing protein damage may also be important in retarding the aging process.[31]

Although tissue and cellular levels of melatonin have not been defined, the majority of the evidence supports the hypothesis that these are higher than plasma levels. Thus, much higher levels of melatonin have been described in the gastrointestinal tract, cerebrospinal fluid (CSF), bone marrow cells and bile.[3,32,33] Nonetheless, even physiological concentrations of melatonin in the blood, although in the pico- to nanomolar range, contribute to the total antioxidant capacity of serum,[34] thus demonstrating that melatonin, at plasma concentrations, may exert some antioxidant function. Additionally, rats which live with a melatonin deficiency throughout life due to pinealectomy exhibit an accelerated accumulation of damaged proteins.[35] Keeping in mind that *in vitro* experiments involves the use of very high concentration of pro-oxidant agents (i.e. 40 mM AAPH in the current study), this explains why much higher concentrations of melatonin (or other antioxidants) are required to counteract the toxic effects of peroxyl radicals as in the current study. *In vivo*, however, concentrations of pro-oxidants are obviously much lower, and thus lower levels of antioxidants (including melatonin) may be effective in protecting against protein oxidative damage. Additionally, many studies have shown that melatonin regulates and/or maintains the intracellular concentration of GSH.[36] Consequently, the protective role of melatonin *in vivo* may be enhanced due to its ability to maintain high GSH levels. Finally, the scavenging of melatonin is not confined to this molecule; thus, the metabolites that are formed when melatonin scavenges radicals, like cyclic 3-hydroxy-melatonin or N-acetyl-N′-formyl 5-methoxykynuramine (AFMK),[37] are themselves scavengers, thereby enhancing melatonin's protective effect.[38]

Catalase is one of the most important antioxidant enzymes used by cells to defend against the toxic effects of H_2O_2 which is generated by various reactions and/or environmental agents, or by the action of superoxide dismutase (SOD) enzymes while detoxifying $O_2^{\bullet -}$.[39] These detoxifying enzymes constitute the first line of defense against the formation of most highly reactive oxygen-derived free radical, the $^{\bullet}OH$. These enzymes cooperate to ensure global cellular protection.[40] To our knowledge, this is the first report describing the damaging effects of the alkyl-peroxyl radicals generated by the azo initiator AAPH to modify the structure and function of the enzyme catalase. The carbon-centered radicals formed from AAPH or any other endogenous sources are able to react with lipids to yield the lipid peroxyl radical via the chain reaction. The radicals initially formed are able to eventually damage any macromolecule including DNA and proteins.[41]

It has been recently shown that Cu, Zn-SOD is also altered and inactivated by AAPH. This suggests that peroxyl/alkyl-peroxyl radicals markedly increase cellular damage by inactivating key antioxidant enzymes via their oxidative modification. In this case, the toxic insult of free radicals is enhanced by the inactivation of one of the main protective cellular systems, namely the synergistic action of the SODs and catalase. There is little information related to the possible oxidative inactivation of other antioxidant enzymes, i.e. GSH peroxidase (GPx) and GSH reductase (GRd), by peroxyl radicals or by lipid peroxidation-derived products. However, both GPx and GRd are inactivated by metal catalyzed oxidation and $^{\bullet}OH$,[42] so the possibility exists that free radicals generated during the breakdown of lipids can indeed inactivate all major antioxidant enzymes. It is well documented that oxidative damage and aging are associated with a decline in antioxidant enzyme activities.[43,44] These reductions may be a consequence of their oxidative modification over time. Additionally, this may explain the existence of a toxic threshold above which cells do not exhibit a defensive response since their protective effects are lost due to their inactivation. Collectively, the current and previously published

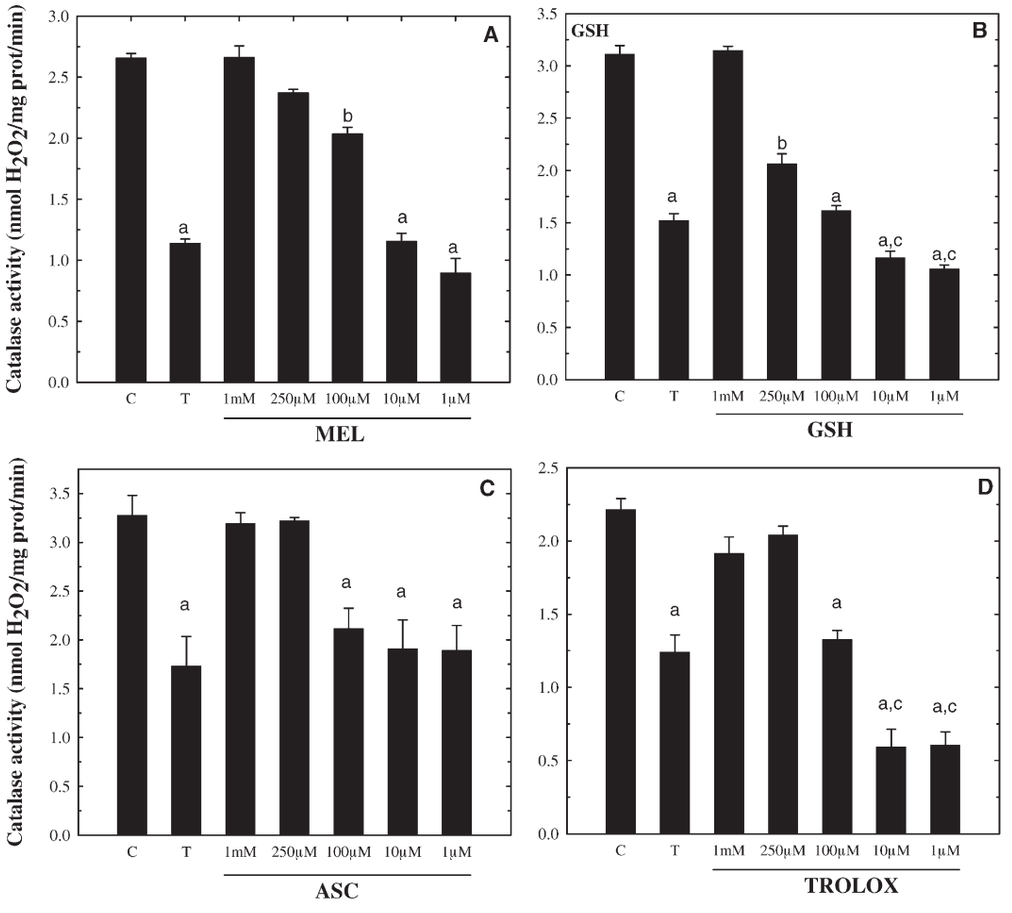

FIGURE 5 Effect of antioxidants on the catalase activity after AAPH treatment. Catalase (0.5 mg/ml) was left untreated (C, control) or incubated with 40 mM of AAPH (T) for 2 h at the concentrations indicated in the presence of antioxidants melatonin (A), GSH (B), ascorbate (C) or trolox (D). For measurement of catalase activity, H_2O_2 determination using horseradish peroxidase and phenol red was used (for details, see Materials and Methods section). Results show the mean ± SEM of three independent experiments. (a) $P < 0.05$ vs. remain of groups; (b) $P < 0.05$ vs. control group (C); (c) $P < 0.05$ vs. treated group without antioxidants (T).

results emphasize the importance of antioxidants in general and melatonin in particular in the maintenance of antioxidant enzyme activity.

Thermal decomposition of the water-soluble azo compound AAPH can initiate the lipid peroxidation chain reaction[20,41] which leads to formation of lipid peroxidation end-products including *n*-alkanals, 2-alkenals, hydroxyl-alkenals and MDA.[13] It is well established that some of these end-products, e.g. HNE and MDA, cause protein damage by reacting with lysine amino groups, cystein sulfhydryl groups and histidine imidazole groups;[13,45] this results in the destruction of the natural biological activities of the enzymes.[46,47] These observations have encouraged many groups to study the "oxidized lipid-protein" complexes, as well as the ability of antioxidants to interact with these agents and to decrease this damage. Accordingly, use of AAPH provides an excellent and controlled model for peroxyl radical-mediated oxidation of proteins.[48] It is well known that vitamin E or trolox, but not vitamin C, can inhibit AAPH-induced lipid peroxidation.[49] However, the current study suggests that melatonin is more effective than the other antioxidants in preventing the protein damage due to peroxyl radicals. The actions of trolox in red blood cell ghost membrane proteins are dependent on the phase in which the free radicals are produced. Therefore, when radicals are generated in the aqueous phase, vitamin E is not particularly effective;[50] this may partially explain the higher efficacy of melatonin and the protection afforded by vitamin C in this situation. The current findings may indicate that, *in vivo*, the melatonin's blockade of protein oxidation by lipid peroxidation end-products may be important, although this issue should be further investigated. In addition to the data presented here, it was recently found that melatonin (100 μM) protects red blood cell proteins against the toxicity of MDA, preventing mainly the protein cross-linking caused by MDA, as it occurred in our study with AAPH-induced protein modification.[51] Marshall and co-workers[52] have shown that melatonin is an excellent scavenger

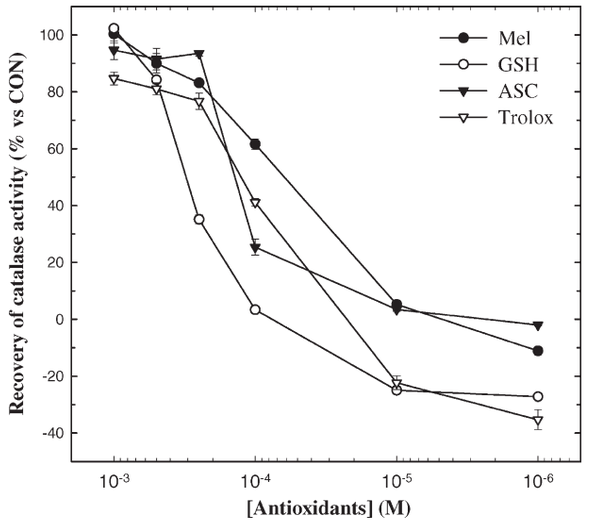

FIGURE 6 Comparative effect of antioxidants on the catalase activity after AAPH treatment. Catalase (0.5 mg/ml) was left untreated or incubated with 40 mM of AAPH for 2 h at the concentrations indicated in the presence of antioxidants melatonin (A), GSH (B), ascorbate (C) or trolox (D). For measurement of catalase activity, H_2O_2 determination using horseradish peroxidase and phenol red was used (for details, see Materials and Methods section). Data were standardized to the untreated control (100% activity) and the percentage of recovery with respect to the AAPH treated group is represented. Results show the mean ± SEM of three independent experiments. Data are derived from a different set of experiments from those shown in Fig. 5.

of the peroxyl radical $CCl_3O_2^{\bullet}$, better than trolox, lipoic acid or propyl gallate. This suggests that melatonin may prevent both alkyl-peroxyl and lipid peroxidation end-product mediated protein damage.

The relative importance of carbonyl formation in the inactivation of enzymes remains a matter of controversy, as this accounts for only a portion of the oxidative modifications induced by free radicals in proteins.[20–22] The carbonyl content is increased in several tissues during aging,[20] but the increases in carbonyls are not necessarily associated with decreases in enzyme activity.[53] This is consistent with the current observations in which we found enzyme activity to be reduced after 2 h incubation, while the carbonyl content only increased dramatically after 8 h. As is the case with Cu,Zn-SOD or gluthamine synthetase activities after AAPH treatment,[54,55] the reduced activities may be due to oxidation of critical residues rather than to their carbonyl content. The antioxidants used in the current study were better at protecting protein structure and enzyme activity than they were at decreasing carbonyl formation (Figs 2–4), suggesting that other oxidative processes may be involved in catalase inactivation. Nevertheless, carbonyl content is still useful as an indirect means to estimate global protein oxidation and therefore antioxidant protection.[56]

In most of the oxidative damage models investigated to date, melatonin seems to have a behavior similar to trolox,[27,29,57,58] with the latter being slightly better in protecting against lipid peroxidation while the former is more effective in protecting DNA.[59] The results reported here indicate that especially at lower concentrations, melatonin is more effective in protecting protein structure against peroxyl radicals than is trolox. The absence of an effect of GSH and ascorbate at lower concentrations is likely due to the hydrophilic nature of these compounds. The lipophilic character of alkyl-peroxyl radicals generated by AAPH requires high concentrations of these compounds to be effective. On the contrary, both melatonin and trolox act at lower concentrations possibly due to their lipophilicity. Surprisingly, trolox at very low concentrations (Figs 5 and 6) exhibited a "pro-oxidant" effect in terms of enhancing the loss of catalase activity. This was, however, not consistent with the PAGE analysis or carbonyl measurements.

In summary, the results shown here indicate that alkyl-peroxyl radicals oxidize and inactivate catalase and this could lead to a reduction in the antioxidant capacity of cells. The implications of lipid peroxidation end-products in catalase inactivation should be further investigated, as well as the protective role of melatonin. The presence of antioxidants, especially melatonin, prevents catalase inactivation by neutralizing alkyl-peroxyl radicals thus protecting protein structure and enzymatic function. The role of antioxidants may, therefore, be two-fold; in one case they directly protect macromolecules against free radicals and in the other they preserve the cellular enzymes which provide protection against oxygen-based reactants.

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