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SHORT COMMUNICATION

Suppression of Both Basal and Antigen-Induced Lipid Peroxidation in Ring Dove Heterophils by Melatonin

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ABSTRACT. There have been several findings recently concerning melatonin as a free radical scavenger and general antioxidant. For instance, in bird heterophils we found that 100 μM of melatonin decreases superoxide anion levels and modulates superoxide dismutase activity. This paper sought to study the effect of melatonin upon induced oxidative damage in heterophils of the ring dove (*Streptopelia risoria*). The concentration of malonaldehyde (MDA) as an index of induced oxidative damage to lipid membranes was tested by colorimetric assay. A heterophil suspension was co-incubated with and without inert particles (latex beads) as material to be phagocytosed, both alone and in combination with 100 μM of melatonin. Measurements were made at the basal time (0 min), as well as at 15, 30, 45, and 60 min. Protein concentrations were determined by a standardized method using bovine serum albumin as standard. Results are expressed as nmol MDA/mg prot. Melatonin clearly reduced the production of MDA, an index of lipid peroxidation. It also annulled the enhancement of MDA levels produced by latex beads. Both effects were observed at all the times studied. In conclusion, our findings again show that the neurohormone melatonin could be useful as an effective pharmacological antioxidant. BIOCHEM PHARMACOL **58**;8:1301–1306, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. melatonin; lipid peroxidation; heterophils; ring dove

The principal pineal hormone, melatonin, is a highly evolutionarily conserved molecule which participates in many important physiological functions. In mammals, these functions notably include actions upon seasonal reproduction [1, 2] circadian rhythm, sleep, mood, tumor growth, and aging [3, 4], and recently effects upon the immune system have also aroused considerable interest [5–8]. In birds, where melatonin is closely associated with circadian rhythms [9], the avian pineal gland has been demonstrated to be a lymphoid organ [10, 11], and administration of exogenous melatonin or *in vitro* incubation with the hormone can influence the antigen-specific and non-specific immune response [12, 13]. Accumulated evidence shows that melatonin's actions may be mediated by membrane receptors [14] and nuclear receptors [15], or via direct intracellular actions due to its highly lipophilic nature [16]. The finding that removal of the pineal gland in the ring dove resulted in an increase in the levels of superoxide anion [17], and our recent observations that *in vitro* incubation with 100 μM of melatonin induces a decrease in superoxide anion levels and modulates superoxide dismutase activity [18], are of particular interest, as recent observations in mammals have indicated that melatonin is a potent radical scavenging antioxidant [19–25]. Since it is possible that one pineal-mediated effect upon the immune system may be a direct action of melatonin on the phagocytic biochemical process, and considering that free radicals have been postulated to be the major mediator of tissue injury in several model systems [26–28], we sought to examine whether melatonin *in vitro* may affect lipid peroxidation, which is frequently used as an index of cellular oxidative damage, in heterophil leukocytes isolated from the ring dove.

MATERIALS AND METHODS

Animals

Male and female ring doves (*Streptopelia risoria*) at least 2 years old were used in the study. Birds were housed in isolation in cages measuring 40 × 40 × 45 cm and fed *ad lib*. The daily lighting pattern was 14 hr light: 10 hr dark with lights on at 8 a.m. The temperature was maintained at 22 ± 2°. Birds were chosen at random and a single sample was taken from each bird.

Isolation of Heterophil Leukocytes

Heterophil leukocytes were obtained from 1 mL of heparinized blood drawn from the brachial vein (at between 10 and 11 a.m.), followed by centrifugation at 600 × *g* for 15 min in a gradient using Histopaque (0.9 mL of 1119 + 0.9 mL of 1077, Sigma). The heterophils were then washed in PBS and adjusted to 5×10^6 cells/mL of medium.

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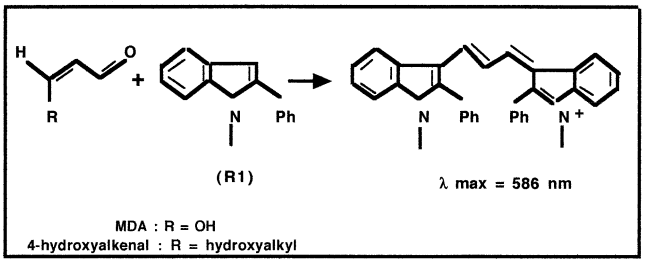

SCHEME 1. A schematic representation of the reaction of two molecules of the reagent R1 (Bioxytech) with either one MDA or one 4-hydroxyalkenal molecule yielding a stable chromophore with maximal absorbance at 586 nm.

Melatonin

N-Acetyl-5-methoxytryptamine (Sigma) was prepared in PBS solution, starting from a base solution of 1 g/100 mL which was dissolved by heating and stirring, followed by diluting working solutions to 100 μM. The choice of this concentration was based on our preliminary studies [18], where we had observed that this dose affects some phagocytic biochemical processes. All determinations were accompanied by a control sample free from hormone.

Measurement of Lipid Peroxidation

LPO* is a well-established mechanism of cellular injury in both plants and animals. This process leads to the destruction of membrane lipids and production of lipid peroxides and their by-products, such as aldehydes. MDA and 4-hydroxyalkenals, such as 4-hydroxy-2(E)-nonenal (4-HNE), are end products derived from the breakdown of polyunsaturated fatty acids and related esters. Measurement of such aldehydes provides a convenient index of lipid peroxidation [29]. In spite of the utility of MDA and 4-HNE as markers of lipid peroxidation, no simple and reliable assay has existed until now. The LPO-586 assay is based on the reaction of a chromogenic reagent, R1 (Bioxytech), with MDA and 4-hydroxyalkenals at 45°. Both MDA and the 4-hydroxyalkenals react with 2 molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm (Scheme 1). The light wavelength and low temperature of incubation (45°) used in this procedure eliminate interference and undesirable artifacts.

The cell precipitate (5×10^6 cells/mL) was separated into aliquots of 300 μL per tube. One tube contained only cells (negative control); a second cells with latex beads (1.091-μm diameter, at 1% in PBS, Sigma), constituting the positive control; a third cells with melatonin (100 μM); and the last cells with a combination of melatonin and latex beads. The tubes were incubated in a thermal bath at 37° for different times (15, 30, 45, and 60 min). The samples were subjected to ultrasound (70 W, three cycles of 30 sec) and centrifuged at 15,000 × *g* (10 min, 0°). All trials were accompanied by a basal sample (t = 0 min) which contained 300 μL cell suspension plus PBS. Subsequently, 200 μL samples were taken from each supernatant (200 μL buffer for the negative control), and 650 μL of diluted R1 (18 mL of R1 + 6 mL of 100% ethanol, Bioxytech) was added. Then, 150 μL of HCl (37% by weight) was added to stop the reaction, followed by stirring to a vortex for 3–4 sec and incubation in a thermal bath at 45° (60 min). The sample was then transferred to a quartz cuvette, and the absorbance of the stable chromophore produced as a function of the lipid peroxidation was measured at 586 nm. All trials were carried out in triplicate for each sample and included a blank for each series of assays ([aldehyde] = 0) by replacing the sample with buffer. Blank absorbance was subtracted from sample absorbance for calculations. The LPO-derived aldehydes are covalently trapped in the form of Schiff bases by the amino groups of proteins. Consequently, the method principally measures the free MDA in biological samples. In addition, melatonin, latex, and a combination of these substances were tested with the standard of the Bioxytech kit to evaluate any potential interference between these chemicals and the kit reagents.

Total Protein Levels

The levels of cell lysate total protein were measured using the Bradford protein assay [30] (Sigma). To 100 μL of cellular lysate in PBS was added 5 mL of Bradford reagent (Coomassie brilliant blue G-250 0.01%, ethanol 4.7%, orthophosphoric acid 8.5%, diluted in distilled water). The contents of the tubes were mixed on a rotary mixer. After 15 min, absorbances were read at 595 nm using a tube containing PBS plus Bradford reagent as the blank (unknown samples were assayed in duplicate). The concentration of total protein was calculated by means of a standard curve with bovine albumin (0.05–0.4 mg/mL, 2 mg/mL; Sigma) and the results expressed as mg of protein/mL.

Calculation of Concentrations

The following equation gives the concentration (M) of MDA in the sample:

$$[\text{MDA}] = (A - Ao) \times 5/\epsilon$$

Here, A is the absorbance in the presence of sample, Ao is the absorbance in the absence of the sample, 5 is the sample dilution factor in the cuvette (200 μL of sample in a total volume of 1 mL), and ε is the apparent molar extinction coefficient obtained from the standard curve using S2 (solution of 10 mM 1,1,3,3-tetramethoxypropane in 20 mM Tris–HCl buffer, pH 7.4, 0–20 μM; Bioxytech) diluted 100 times with PBS. Results are expressed as nmol MDA/mg prot.

* *Abbreviations:* LPO, lipid peroxidation; MDA, malonaldehyde; and R1, 10.3 mM of *N*-methyl-2-phenylindole, in acetonitrile.

TABLE 1. Effect of melatonin (100 μM) upon lipid peroxidation in ring dove heterophils

Time (min)	Basal (t = 0 min)	Control	Latex	Melatonin	Melatonin + Latex
	(nmol MDA/mg prot)				
15	5.56 ± 0.02	5.61 ± 0.12	6.48 ± 0.17*†	2.30 ± 0.17*†≠	5.89 ± 0.33*≠§
30	5.46 ± 0.28	6.29 ± 0.32*	8.51 ± 0.44*†	4.62 ± 0.23*†≠	6.27 ± 0.33*≠§
45	5.04 ± 0.36	5.60 ± 0.40*	7.17 ± 0.52*†	3.79 ± 0.27*†≠	4.69 ± 0.34*†≠§
60	5.47 ± 0.36	5.43 ± 0.36	6.39 ± 0.41*†	3.19 ± 0.21*†≠	4.63 ± 0.31*†≠§

Each value represents the mean ± SD of ten determinations performed in triplicate.
$P < 0.05$*, †, ≠, §: * = with respect to basal; † = with respect to control; ≠ = with respect to latex; § = with respect to melatonin.

Statistical Analysis

All data are expressed as means ± standard deviation of the mean for the number of samples assayed. This number (N) is indicated for each case. Results were analyzed by using the non-parametric ANOVA-Scheffe *F*-test to compare differences between groups, followed by a Student's *t*-test. Only values with $P < 0.05$ were accepted as significant.

RESULTS

The lipid peroxidation (nmol MDA/mg prot) in heterophils under different situations (with and without latex beads and/or melatonin) and at different times of incubation (15, 30, 45, and 60 min) are summarized in Table 1. As can be seen, there was a significant increase ($P < 0.05$: *) in the MDA concentration at all the times studied for the latex beads, as well as at 15 and 30 min in the melatonin + latex group, with respect to the values obtained at the basal time. In control samples, only at 30 and 45 min was there a significant increase compared with that at the basal time. On the contrary, after 45 and 60 min of incubation in the melatonin + latex group, there was a significant decrease ($P < 0.05$: *) in the MDA concentration compared with that at the basal time. In addition, at all the times studied and with respect to the control group, there was a significant increase ($P < 0.05$: †) in production of MDA in heterophils incubated with latex beads, and a significant decrease ($P < 0.05$: †) when the phagocytic cells were incubated in the presence of both the pineal hormone alone and when melatonin was in combination with latex, although in this last group only at 45 and 60 min of incubation. When the heterophils were incubated with melatonin or with melatonin + latex, a significant decrease ($p < 0.05$: ≠) was observed with respect to the values obtained in the presence of latex beads alone. At all the times studied, the values obtained with melatonin + latex were higher ($P < 0.05$: §) than obtained in the presence of melatonin alone.

Figure 1 shows the comparison study of the values obtained in each experimental group at the different incubation times. The concentration of MDA (nmol/mg prot) is expressed as a percentage of its basal value (t = 0 min). In all the experimental groups, there were changes in the lipid peroxidation with time. The maximum levels of peroxidation were observed after 30 min of incubation and the minima at 60 min of incubation in the control, latex bead, and melatonin + latex groups, although these levels were only significant in the last group. In contrast, in the samples with melatonin alone the lowest levels of peroxidation were observed at 15 min.

In summary, the highest values of lipid peroxidation were obtained when the heterophils were incubated in the presence of latex beads and the lowest values after incubation with the melatonin hormone. Melatonin annulled the latex-induced enhancement of MDA production.

DISCUSSION

In ring dove heterophils, we have found that 100 μM of melatonin controls superoxide anion levels and modulates superoxide dismutase activity, both in the presence and absence of particulate antigen, which is probably due to the action of this hormone as a scavenger of superoxide anion [18]. Similarly, this neurohormone has been shown to be a more efficient scavenger of the two highly toxic hydroxyl and peroxyl radicals than other known compounds such as vitamin E or vitamin C, mannitol, and glutathione [7, 25, 31], in addition to preventing the toxicity of singlet oxygen [32]. Melatonin also appears to affect the activity of such antioxidant enzymes as superoxide dismutase in our case, or glutathione peroxidase as has been indicated by Reiter [32]. All of this is in accordance with the idea suggested previously by other authors [19, 33] that this hormone is a free radical scavenger *in vitro* and an antioxidant *in vivo*. The exposure of biological membranes to oxidative stress results in a progressive degeneration of membrane structure and loss of integrity. Lipid peroxidation is a process that leads to the destruction of membrane lipids and production of lipid peroxides and their by-products, such as aldehydes [29, 34]. The ability of melatonin to protect against latex bead-induced toxicity *in vitro* was tested here. The concentration of MDA was used as an index of induced oxidative damage to lipid membranes.

The data presented here show that, in ring dove heterophils, lipid peroxidation was clearly reduced by melatonin. This neurohormone alone was effective in reducing

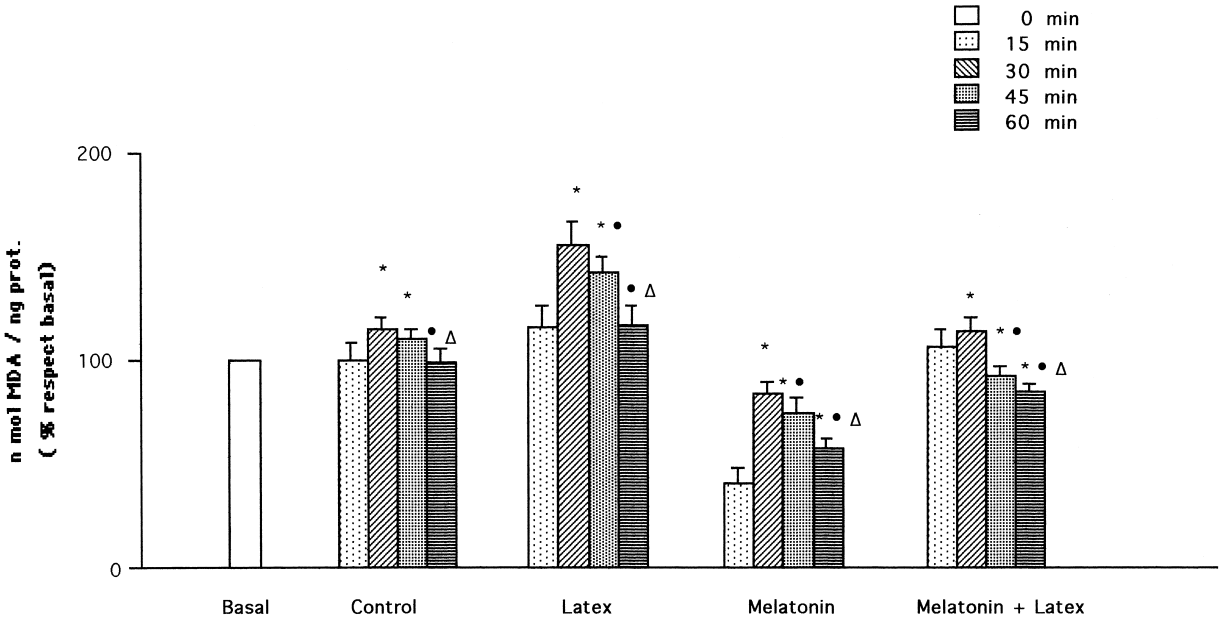

FIG. 1. Changes with time in lipid peroxidation (nmol MDA/mg prot) in heterophils from *Streptopelia risoria*. Each value represents the mean ± SD of ten determinations performed in triplicate. The results are expressed as % with respect to their basal value (0 min). *$P < 0.05$ with respect to 15 min; (●) $P < 0.05$ with respect to 30 min; and (△) $P < 0.05$ with respect to 45 min. Latex: samples incubated in the presence of latex beads. Melatonin: samples incubated in the presence of 100 μM of the hormone. Melatonin + latex: samples incubated in the presence of both melatonin and latex.

basal levels of peroxidation in heterophils at all the incubation times, the lowest levels of lipid peroxidation being at 15 min. In addition, melatonin depressed the increased levels of MDA after latex bead treatment. Thus, melatonin annulled the latex bead-induced enhancement of MDA production with a time-dependent effect. At shorter times of incubation in the presence of the hormone, the MDA levels, which had been raised by the latex beads, returned to control values. At longer times of incubation, the MDA levels in the melatonin + latex group were lower than in the controls. This could principally be due to the hormone's possibly acting alone at shorter times of incubation when the other antioxidant mechanisms of the phagocytic cells might be only partially activated. On the contrary, at the longest times of incubation the results could be due to a sum of the effect of melatonin together with the several enzyme systems and cellular components that protect against activated forms of oxygen after the respiratory burst, such as superoxide dismutase, myeloperoxidase, catalase, and vitamins C and E.

The results of this study demonstrate that melatonin effectively protects against both basal and latex bead-induced toxicity. These findings are consistent with the data reported by other authors on the protective effect of the neurohormone melatonin against both basal and stress-related lipid peroxidationin in other *in vitro* models using bacterial lipopolysaccharide [35] or H_2O_2 [36] to induce toxicity. This protective effect of melatonin against lipid peroxidation may be related to its known ability to scavenge free radicals and function as an antioxidant, as has been indicated previously by other authors [19, 25, 37–39]. Also, there is now evidence that melatonin may reduce lipid peroxidation in cellular membranes by processes independent of its free radical scavenging activity. Thus, its ability to stabilize cellular membranes could make their intrinsic lipids more resistant to peroxidative processes [40, 41].

The pharmacological melatonin dose used in this *in vitro* study, as well as the doses utilized by other authors in both *in vivo* and *in vitro* studies [16, 21–25, 37, 42], exceed the low physiological concentrations which are normally found in plasma and are much higher than peak nighttime serum concentrations in both mammals (animals and humans) [4, 43] and birds [44]. In addition, because of the magnitude of the oxidative challenge in the present experimental situation, no antioxidant at physiological levels would be expected to prevent lipid peroxidation, and only pharmacological levels of antioxidants appear to prevent the oxidative damage induced by "pharmacological levels" of free radicals.

In summary, our results are consistent with the high lipid solubility of melatonin and with other findings, both *in vivo* and *in vitro* [45], which demonstrate the ability of this indolamine at pharmacological doses to reduce oxidative damage to lipids. Whether physiological concentrations of melatonin are significant in reducing lipid peroxidation is unknown. In addition, we do not presently have full knowledge of intracellular melatonin levels; like indole, melatonin might act intracellularly to exert its pharmacological and/or physiological effects. We believe that future investigations may provide answers as to whether melato-

nin's actions are merely pharmacological or also have physiological relevance.

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