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Melatonin protects human red blood cells from oxidative hemolysis: New insights into the radical-scavenging activity

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Abstract: Antioxidant activity of melatonin in human erythrocytes, exposed to oxidative stress by cumene hydroperoxide (cumOOH), was investigated. CumOOH at 300 μM progressively oxidized a 1% suspension of red blood cells (RBCs), leading to 100% hemolysis in 180 min. Malondialdehyde and protein carbonyls in the membrane showed a progressive increase, as a result of the oxidative damage to membrane lipids and proteins, reaching peak values after 30 and 40 min, respectively. The membrane antioxidant vitamin E and the cytosolic reduced glutathione (GSH) were totally depleted in 20 min. As a consequence of the irreversible oxidative damage to hemoglobin (Hb), hemin accumulated into the RBC membrane during 40 min. Sodium dodecyl sulfate (SDS) gel electrophoresis of membrane proteins showed a progressive loss of the cytoskeleton proteins and formation of low molecular weight bands and protein aggregates, with an increment of the intensity of the Hb band. Melatonin at 50 μM strongly enhanced the RBC resistance to oxidative lysis, leading to a 100% hemolysis in 330 min. Melatonin had no effect on the membrane lipid peroxidation, nor prevented the consumption of glutathione (GSH) or vitamin E. However, it completely inhibited the formation of membrane protein carbonyls for 20 min and hemin precipitation for 10 min. The electrophoretic pattern provided further evidence that melatonin delayed modifications to the membrane proteins and to Hb. In addition, RBCs incubated for 15 min with 300 μM cumOOH in the presence of 50 μM melatonin were less susceptible, when submitted to osmotic lysis, than cells incubated in its absence. Extraction and high-performance liquid chromatography (HPLC) analysis showed a much more rapid consumption of melatonin during the first 10 min of incubation, then melatonin slowly decreased up to 30 min and remained stable thereafter. Equilibrium partition experiments showed that 15% of the melatonin in the incubation mixture was recovered in the RBC cytosol, and no melatonin was extracted from RBC membrane. However, 35% of the added melatonin was consumed during RBC oxidation. Hydroxyl radical trapping agents, such as dimethylsulfoxide or mannitol, added into the assay in a 1,000 times molar excess, did not vary melatonin consumption, suggesting that hydroxyl radicals were not involved in the indole consumption. Our results indicate that melatonin is actively taken up into erythrocytes under oxidative stress, and is consumed in the defence of the cell, delaying Hb denaturation and release of hemin. RBCs are highly exposed to oxygen and can be a site for radical formation, under pathological conditions, which results in their destruction. A protective role of melatonin should be explored in hemolytic diseases.

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Introduction

In recent years, plenty of experimental data provided unequivocal evidence that a number of free radicals, including reactive oxygen species, may be formed in biological systems, either endogenously or from exogenous sources [Davies, 1995]. Such reactive species may bring about oxidative damage to virtually all cell compartments, eventually leading to various pathologies and aging [Harman, 1993; Beckman and Ames, 1998]. These studies prompted research on physiological antioxidant systems and molecules, and stimulated the development of natural or synthetic compounds that prevent oxidative stress and damage mediated by an enhanced formation of free radicals [Rice-Evans and Diplock, 1993; Halliwell, 1996].

After the discovery of its radical-scavenging properties [Tan et al., 1993], melatonin has been considered as a putative biological antioxidant, but it has been questioned as to whether it may have a real antioxidant function under pysiological in vivo conditions [Loffler, 1996; Marshall et al., 1996; Brzezinski, 1997; Reiter et al., 1997a,b]. Although preliminary evidence in vivo had suggested that endogenously produced melatonin was able to limit oxidative damage by the carcinogen safrole [Tan et al., 1994], comparable effects in pure chemical systems or in cell preparations were not obtained with physiological amounts of melatonin. Molecular mechanisms and conditions of actions of melatonin remain to be better clarified. Possibly, interactions contributing to the antioxidant effects in vivo may be lost in in vitro experiments. As observed in vitro, melatonin is an electron donor. A number of electrophilic compounds, such as the hydroxyl radical, Fe^{3+}, or carbon-centered radicals may act as acceptors in one-electron transfer reactions, which convert the indolamine to the indolyl cation radical [Poeggeler et al., 1994]. Reactivity of melatonin with oxygen-centered radicals, such as peroxyl [Pieri et al., 1994] or alkoxyl radicals [Scaiano, 1995], as well as a moderate activity towards lipoperoxyl radicals [Marshall et al., 1996; Livrea et al., 1997], has also been demonstrated. Therefore, the ability to scavenge a broad spectrum of radicals could allow melatonin to behave as an antioxidant in various and possibly complex ways.

We decided to establish a biological model system in which various oxygen- and carbon-centered radicals were generated, to investigate the antioxidant capacity of melatonin in such a context. Erythrocytes (red blood cells, RBCs) have been a traditional target for studying oxidative damage. Exposed to high oxygen tensions and rich, as they are, in iron, a transition metal promoting the formation of oxygen free radicals, erythrocytes may be easily susceptible to oxidation and eventual hemolysis. The mechanism of hemolysis in vitro is a consequence of the system used to induce the stress. Oxidant damage of external origin or internal free radical generation may have different effects. Cumene hydroperoxide (cumOOH), a lipophilic iron-dependent oxidant, preferentially locates in the membrane and induces a primary extensive hemoglobin (Hb) oxidation [Maples et al., 1990; Van den Berg et al., 1992]. Then, a process is set in motion in which cumene- and globin-derived radicals [Tornalley et al., 1983; Davies, 1988] and precipitation of Heinz bodies onto the RBC membrane bring about membrane oxidative modifications, leading to hemolysis.

The effect of melatonin in human RBCs exposed to oxidative stress by cumOOH has been evaluated by different means, i.e. extent of hemolysis, evaluation of membrane lipid and protein oxidation products, electrophoretic pattern of membrane proteins, relationships with the erythrocyte GSH, and formation of degradation products (hemin) of Hb.

Materials and methods

Chemicals

CumOOH, α-tocopherol, melatonin, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), tetraethoxypropane (TEP), 2,4 dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitro benzoic acid) (DTNB), dimethylsulfoxide (DMSO), mannitol, tris(hydroxymethyl) aminomethane (TRIS), pyrogallol, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, tetramethylethylenediamine (TEMED), and Coomassie blue were from Sigma (St. Louis, MO). All other chemicals were of research highest purity grade. Ion-free water and buffers used throughout this study were filtered through Chelex-100 (Sigma), and suitable plastic labware was used to avoid the effect of adventitious metals.

Red blood cells

Blood samples were obtained from apparently healthy individuals by venipuncture, with informed consent. Ethylenediaminetetraacetic acid (EDTA) (1 mg/mL blood) was used as anticoagulant. RBCs were sedimented at 1,000g for 10 min and washed three times with phosphate-buffered saline (PBS), pH 7.4. Supernatant and buffy coat were carefully removed by aspiration after each wash.

Oxidation of red blood cells by cumene hydroperoxide

A 1% suspension of RBCs in PBS (HT 1%; HbO_2 168 ± 2 μM per heme group) was incubated with cumOOH at 37°C either in the presence or the absence of melatonin. Both melatonin and cumOOH were added to RBC suspensions as ethanol solution. The volume added never exceeded 0.5% of the total incubation volume. The extent of hemolysis was determined at known time intervals, as follows. A volume of the incubation mixture was diluted with 40 volumes of PBS and centrifuged at 1,000g for 10 min to precipitate cells. The absorbance of the supernatant was then evaluated at 540 nm. Similarly, a volume of the incubation mixture was treated with 40 volumes of 5 mM sodium phosphate buffer, pH 7.4 (hypotonic PBS), to yield complete hemolysis, and supernatant after centrifugation was evaluated spectrophotometrically at 540 nm. The percentage of hemolysis was calculated from the ratio of the absorbances.

Measurement of lipid peroxidation

Malondialdehyde (MDA) was measured as an index of lipid peroxidation. Briefly, 0.5 mL of the incubation mixture were mixed with 1.5 mL of H_2O, 5 μL of 40 mM BHT, and 5 μL of 1 M NaOH. After cell homogenization, 250 μL of 0.6% TBA in 35% $HClO_4$ were added and the suspension heated for 60 min. Then, samples were cooled, centrifuged at 1,000g for 10 min, and the MDA–TBA adduct in the supernatant was separated by isocratic high-performance liquid chromatography (HPLC), performed on a Supelco Supelcosil LC-18 column (0.46×25 cm) (Bellafonte, PA). Eluent was 40% methanol in 50 mM potassium phosphate buffer, pH 6.8, at 1.5 mL min^{-1}. The MDA–TBA adduct was revealed spectrophotometrically at 532 nm and quantified by reference to a calibration curve of TEP submitted to the TBA colorimetric procedure.

Osmotic fragility

Aliquots (200 μL) of RBC suspension (1% HT) were added to a series of test tubes containing 1.8 mL of diluted saline phosphate buffer, pH 7.4 (54–40%), and centrifuged at 1,000g for 10 min. Then, the supernatants were assayed for the degree of hemolysis by comparing the absorbance at 540 nm with that of 200 μL of RBC suspension treated with 1.8 mL hypotonic phosphate buffer. The percentage of hemolysis was calculated from the ratio of the absorbances.

Determination of glutathione in red blood cells

Intracellular GSH was determined by titration with DTNB. Briefly, 25 μL of 40 mM BHT were added to 3.0 mL of incubation mixture to stop oxidative reactions. Centrifugation at 1,000g was carried out for 10 min, and 0.5 mL of H_2O were added to the RBC pellet to lyse the cells. Proteins were precipitated by addition of 0.5 mL of a metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.20 g EDTA, and 30 g NaCl in 100 mL of H_2O). After a centrifugation at 3,000g for 10 min, 400 μL of the clear supernatant were combined with 500 μL of 300 mM Na_2HPO_4, pH 8.0, and the absorbance at 412 nm was read against a blank consisting of 400 μL of supernatant and 500 μL of H_2O. Then, 100 μL of a DTNB solution (20 mg DTNB in 100 mL of 1% sodium citrate) were added to the blank and the sample, and the absorbance of the sample was read against the blank at 412 nm, after 5 min at 37°C in a thermostatic cuvette to allow color development. The molar extinction coefficient of GSH is 13,600 cm/M [Hu, 1994].

Vitamin E in red blood cells

Vitamin E (α-tocopherol) was determined by reverse phase HPLC with fluorescence detection (ex 335; em 290). Samples (5 mL) of the incubation mixture were centrifuged at 1,000g for 10 min, after addition of 25 μL of 40 mM BHT. The RBC pellet was resuspended with 1 mL of PBS containing 0.5% pyrogallol, and vitamin E was extracted with 2 mL of absolute ethanol, containing 1% pyrogallol, and 8 mL of petroleum ether. After vortexing, the organic phase was dried under nitrogen, resuspended in a suitable volume of methanol and analyzed by a LC-18 HPLC column, with methanol at 1 mL/min.

Determination of hemin from RBC membranes

The amount of hemin associated with RBC membranes was measured in membrane pellet resuspended in 1% SDS in PBS by spectrophotometry at 408 nm. Concentrations were evaluated from a calibration curve of 2–10 μM pure hemin solutions in the same SDS buffer.

Equilibrium partition studies and melatonin consumption during red blood cell oxidation

RBCs (HT 1%) were incubated at 37°C for 30 min in the presence of 50 μM melatonin. Then, RBCs were sedimented at 1,000g for 10 min and washed

three times with PBS. Melatonin was measured in the soluble and in the total membrane fraction obtained after hemolysis of RBC with hypotonic buffer and precipitation at 110,000g. Aliquots (100 μL) of the RBC cytosol and of the membrane fraction resuspended in PBS (0.2 mg protein/mL), in a total water volume of 300 μL, were extracted with 2 mL of ethyl acetate. After vortexing, the organic phase was gathered and 50 μL of the organic extract were analyzed by a normal phase Supelco Nucleosil-100 HPLC column and eluted with ethyl acetate, according to Vitale et al. [1996]. Fluorimetric detection was with excitation at 285 nm and emission at 345 nm. Quantitation was by reference to a curve constructed with 0.5–5 nmol of melatonin. Melatonin consumption during RBC oxidation in the presence of cumOOH was evaluated by extracting, at appropriate time intervals, 100 μL of the incubation mixture, followed by HPLC analysis, as reported above.

Competition experiments

DMSO or mannitol at 50 mM were included in a incubation mixture containing a 1% suspension of RBCs in hypotonic PBS, in the presence of 300 μM cumOOH and 50 μM melatonin. Melatonin consumption was evaluated, as reported above.

Determination of protein carbonyls in red blood cell membranes

Aliquots (3 mL) of the incubation mixture were mixed with 25 μL of 40 mM BHT. After centrifugation at 1,000g for 10 min, 8 mL of hypotonic PBS were added to the samples to allow the hemolysis takes place. Then, the samples were shaken for 5 min at room temperature and centrifuged at 20,000g for 10 min. The RBC membranes were washed twice with PBS and finally homogenized in 1.0 mL of PBS. The homogenate was divided into equal aliquots (0.5–0.7 mg protein) that were treated with 10% trichloracetic acid (TCA) (w/v, final concentration). One sample was treated with 2 N HCl (tissue blank), and the other was treated with an equal volume of 0.2% DNPH in 2 N HCl (w/v), as reported by Amici et al. [1989]. The samples were incubated at room temperature for 60 min, re-precipitated with 10% TCA (final concentration), and subsequently extracted with ethanol:ethyl acetate (1:1, v/v). The extracts were re-precipitated with 10% TCA, and the pellets were carefully drained and dissolved in 100 mM NaOH. The difference spectrum of the DNPH-treated sample versus the tissue blank was recorded, and the results were expressed as nmol of protein carbonyls/mg membrane protein, based on the absorbance molar coefficient of 22,000 for aliphatic hydrazones at 365 nm [Amici et al., 1989]. Proteins were determined by the Bio Rad colorimetric method, according to Bradford [1976].

Analysis of red blood cell membrane proteins

RBC membranes sedimented at 110,000g for 60 min were mixed with 500 μL of an SDS–TRIS buffer solution [2% SDS, 10% (v/v) glycerol, 0.03% bromophenol blue, 63 mM Tris–HCl, pH 6.8] and put in a boiling water bath for 10 min. SDS polyacrylamide gel electrophoresis (PAGE) was carried out on a 0.75-mm thick slab gel with 4.4 and 10% gels for condensation and separations, respectively, according to the method of Laemmli [1970]. The amount of protein layered was 30 μg. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The gel system was calibrated for molecular weight determination by measuring the migration of standard proteins (range 16–205 kDa).

Results

Effects of melatonin on cumene hydroperoxide-induced hemolysis and melatonin consumption

When intact RBCs are exposed to cumOOH, hemolysis occurs as a consequence of radical production and oxidative damage to cell components. The initial decomposition of peroxide in the presence of heme iron generates methemoglobin (metHb), hydrogen peroxide, and a number of oxyradicals from cumOOH [Tornalley et al., 1983], capable of attacking unsaturated lipids in membranes and reacting with GSH, Hb, and other soluble and membrane proteins [Yamamoto et al., 1985; Van den Berg et al., 1992]. Equations (1) and (2) summarize the formation of oxygen reactive species and cumOOH-derived oxyradicals initiating the oxidative process in RBCs.

$$\mathrm{cumOOH} + \mathrm{HbO_2} \xrightarrow{H^+} \mathrm{cumOO^\bullet} + \mathrm{metHb} + \mathrm{H_2O_2} \quad (1)$$

$$2\mathrm{cumOO^\bullet} \rightarrow \mathrm{cumOOOOcum} \rightarrow 2\mathrm{cumO^\bullet} + \mathrm{O_2} \quad (2)$$

Human RBCs exposed to 300 μM cumOOH underwent hemolysis, the rate of which rapidly increased after 120 min, as measured from the slope of the curve in Fig. 1. Melatonin delayed the onset of hemolysis in a concentration-dependent manner, and the inhibition was extended for more than 180 and 200 min when melatonin concentrations

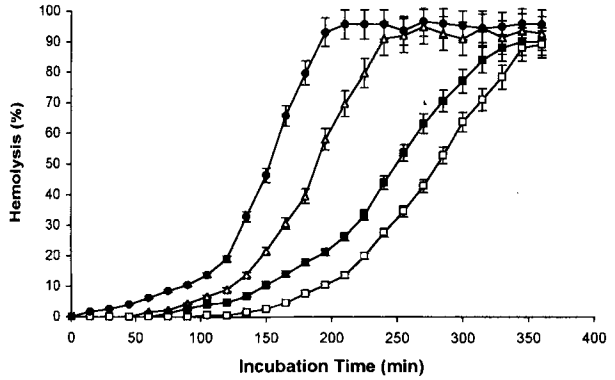

Fig. 1. Effect of melatonin on the cumOOH-induced hemolysis of human erythrocytes. A 1% RBC suspension in PBS was incubated in the presence of 300 μM cumOOH and no melatonin (●), or 20 μM (△), 50 μM (■), or 100 μM (□) melatonin. Hemolysis was measured at time intervals as reported in Materials and methods. Values are the mean ± S.D. of ten separate experiments performed with different RBC preparations.

were 50 and 100 μM, respectively (Fig. 1). All experiments described below refer to assays containing melatonin at 50 μM.

A time-course study reveals a faster consumption of melatonin during the first 10 min of RBC oxidation, then, the indoleamine disappears at a lower rate, until 30 min, when its amount remains stable. The rate of consumption, as measured within the first 10 min of oxidation, was 0.27×10^{7} M/s, and 35% of the melatonin added into the incubation mixture was consumed (Fig. 2). On the other hand, the consumption rate of melatonin was 0.56×10^{7} M/s in experiments in which 1% RBC suspensions were exposed to 450 μM cumOOH, and 75% of melatonin was consumed within 30 min (Fig. 2). This would suggest that consumption of melatonin is relevant to the oxidative reactions triggered by cumOOH into the erythrocyte.

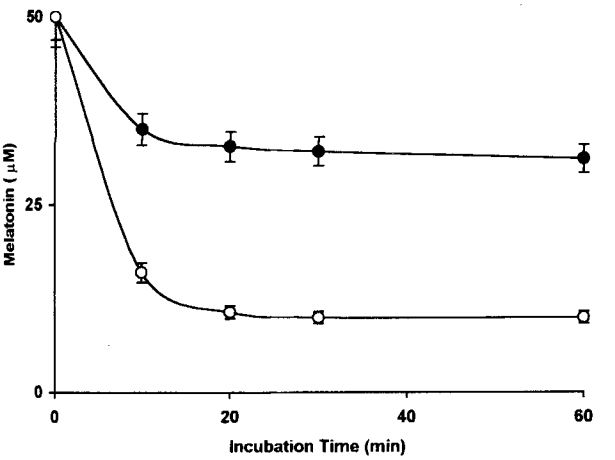

Fig. 2. Time course of melatonin consumption during the oxidation of human RBCs (1% HT) induced by 300 μM (●) or 450 μM (○) cumOOH. Values are the mean ± S.D. of four separate experiments performed with different RBC preparations.

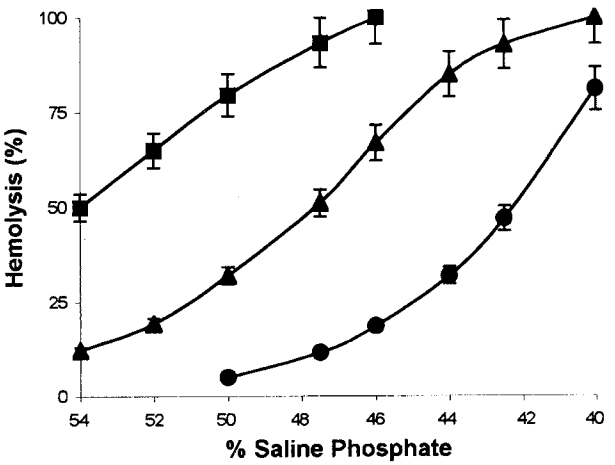

Fig. 3. Susceptibility to osmotic lysis of human RBCs (1% HT) after incubation for 15 min at 37°C in the absence (●) or in the presence (■) of 300 μM cumOOH, or in the presence of 300 μM cumOOH plus 50 μM melatonin (▲). Values are the mean ± S.D. of four separate experiments performed with different RBC preparations.

Equilibrium partition experiments, carried out by incubating 1% RBC suspensions with 50 μM melatonin, in the absence of cumOOH, showed that 15% of the melatonin added into the incubation mixture was recovered into the RBC cytosol, and no melatonin was extracted from the RBC membrane after a 30-min incubation.

Effects of melatonin on the cumene hydroperoxide-induced osmotic fragility of red blood cells

RBCs were incubated in the presence of cumOOH and in the absence and the presence of melatonin, for 15 min at 37°C. Then, the resistance of the erythrocytes to lysis by diluted saline phosphate buffer solutions was assayed. A complete RBC hemolysis occurred with a 46% saline phosphate buffer solution, when RBCs had been treated with cumOOH alone, whereas a 40% saline phosphate buffer solution was needed to cause lysis of RBCs that had been incubated in the presence of cumOOH and melatonin (Fig. 3). The osmotic fragility curve of RBCs exposed to cumOOH and melatonin approached that of control erythrocytes incubated at 37°C in the absence of cumOOH, for 15 min (Fig. 3). This would suggest that, by protecting the erythrocyte against oxidative damage, melatonin makes the cytoskeleton more resistant to mechanical insult.

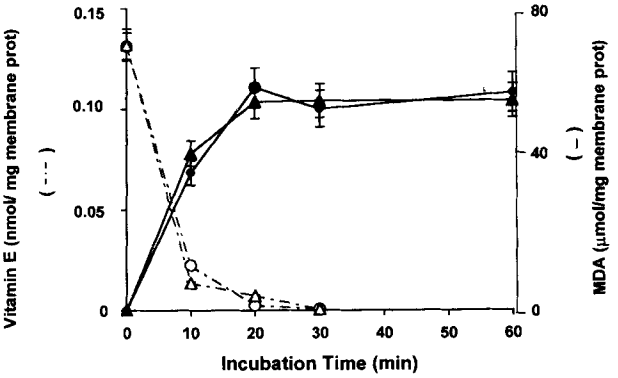

Fig. 4. Vitamin E consumption (open symbol) and malondialdehyde formation (closed symbol) during the cumOOH-induced oxidation of human RBCs in the absence (circle) or in the presence (triangle) of 50 μM melatonin. Values are the mean ± S.D. of five separate experiments performed with different RBC preparations.

Effects of melatonin on cumene hydroperoxide-induced membrane lipid peroxidation

Oxidation of RBC membrane lipids was observed to proceed very rapidly after exposure to cumOOH (Fig. 4). Amounts of MDA as high as 65 ± 5.2 μmol/mg membrane protein were measured after 20-min incubation with the hydroperoxide. Then, MDA remained unchanged for longer incubation times. As a consequence of oxidative stress to the cell membrane, a complete loss of vitamin E is evident after 20 min of incubation (Fig. 4). Melatonin did not change either MDA production or vitamin E consumption (Fig. 4). Incubation of RBCs at 37°C in the absence of cumOOH did not cause a significant loss of vitamin E or production of MDA for 60 min.

Effect of melatonin on cumene hydroperoxide-induced membrane protein carbonyl production

The oxidation of proteins converts the side chain of some of their amino acid residues to carbonyl derivatives [Amici et al., 1989]. Incubation of erythrocytes with cumOOH led to progressive oxidation of membrane proteins. Formation of carbonyls was maximal at 40 min and an amount of protein carbonyls of 30 ± 2.0 nmol/mg membrane protein was measured at that time (Fig. 5). Melatonin completely suppressed formation of protein carbonyls for 10 min and markedly delayed the oxidative damage to membrane proteins within the following 30 min. Thereafter, protein carbonyls were produced with a slower progression than in the absence of melatonin (Fig. 5). Production of protein carbonyls was not observed when RBCs were incubated for 90 min in the absence of cumOOH.

Glutathione analysis

A rapid oxidation of reduced GSH occurs in RBCs under oxidative stress, as a result of a direct radical attack and of repair processes requiring GSH, such as the reduction of oxidized membrane thiol groups. CumOOH induced a rapid consumption of the cytosolic GSH (Fig. 5), and melatonin did not affect this process, indicating that its protection of erythrocytes does not involve cellular GSH homeostasis.

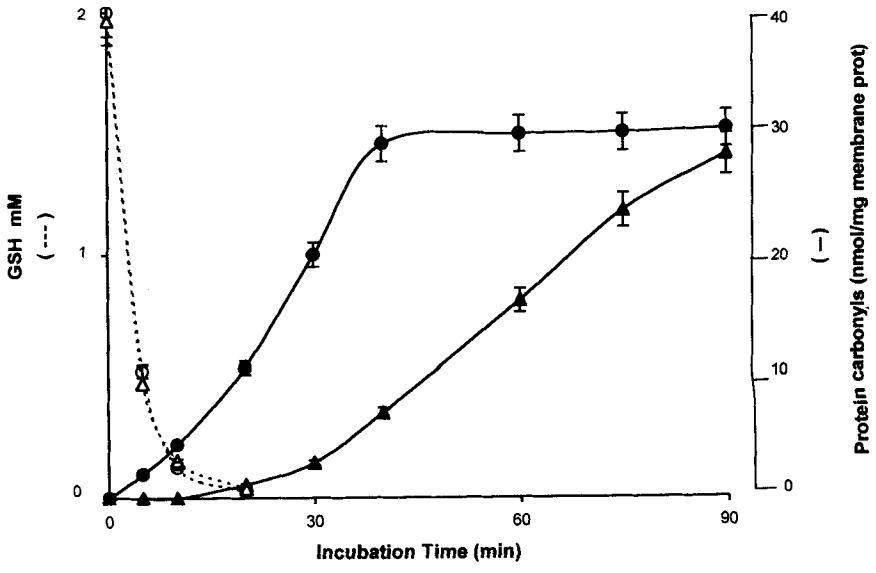

Fig. 5. GSH consumption (open symbol) and membrane protein carbonyl formation (closed symbol) during the cumOOH-induced oxidation of human RBCs in the absence (circle) or in the presence (triangle) of 50 μM melatonin. Values are the mean ± S.D. of five separate experiments performed with different RBC preparations.

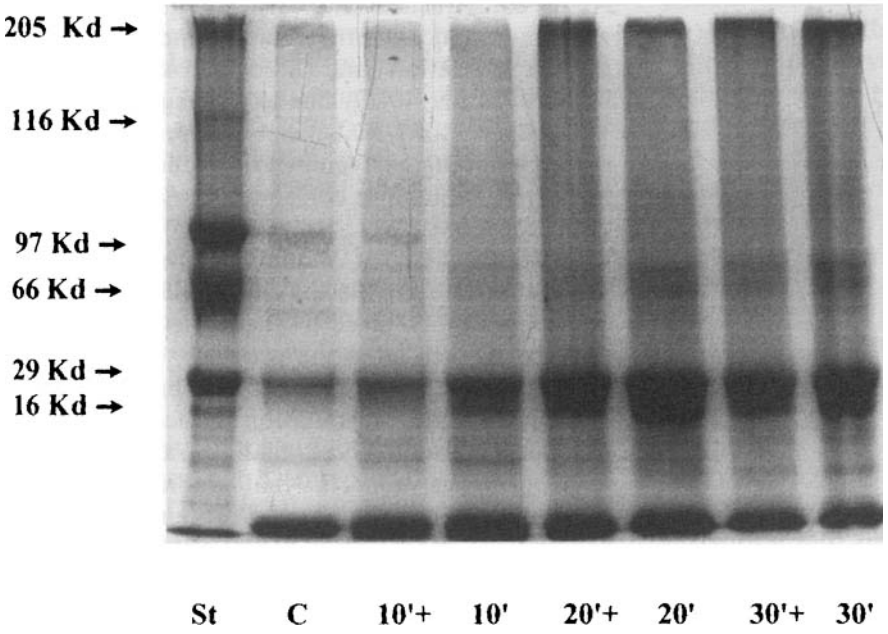

Fig. 6. SDS-PAGE pattern of membrane proteins from RBCs incubated with 300 μM cumOOH for 10–30 min. St, standard proteins and their molecular weights as indicated; C, intact control RBCs; +, in the presence of 50 μM melatonin. The amount of protein layered was 30 μg in each case.

Effect of melatonin on the cumene hydroperoxide-induced changes in the electrophoretic pattern of membrane proteins

The SDS electrophoretic separation of membrane proteins after incubation of RBCs with 300 μM cumOOH in the absence or presence of 50 μM melatonin is shown in Fig. 6. CumOOH leads to loss of α and β spectrin and anion exchange protein bands and to the appearance of high molecular weight aggregates at the top of the gels. In addition, the intensity of the bands relevant to Hb chains increases with the incubation time, and new bands are formed just underneath. Melatonin partly prevents the protein modifications for 30 min.

Effect of melatonin on cumene hydroperoxide-induced hemin accumulation into the red blood cell membrane

Oxidation of Hb in erythrocytes proceeds through a complex series of events, which bring about Hb oxidized intermediates, finally releasing the heme moiety and generating irreversible oxidation products, precipitating on the membrane as Heinz bodies [Winterbourn, 1990]. Heme is highly lipophilic and rapidly partitions into the erythrocyte membrane. The amount of hemin associated with the membrane during the oxidation of the RBC by cumOOH rapidly increases in 10–30 min (Fig. 7). In contrast, in assays in which melatonin was included, a complete inhibition of hemin precipitation onto the RBC membrane was observed for 10 min. Thereafter, the accumulation of hemin occurred at a slower rate than in RBCs treated with cumOOH alone (Fig. 7). Hemin was not extracted from membranes of RBCs incubated at 37°C for 60 min in the absence of cumOOH.

Effects of dimethylsulfoxide and mannitol on melatonin consumption

Melatonin is known as a potent hydroxyl radical scavenger (rate constant 0.6×10^{11} M/s) [Poeggeler et al., 1996]. Oxidation of Hb by cumOOH in our RBC system may favor generation of hydroxyl radicals from heme iron and H_2O_2 [Gutteridge, 1986; Halliwell and Gutteridge, 1989], which can play a role in the oxidation of other cell substrates. If hydroxyl radicals are involved in reactions with

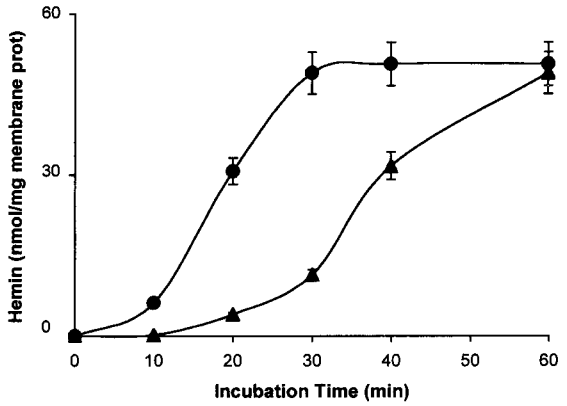

Fig. 7. Accumulation of hemin in membrane during cumOOH-induced oxidation of RBCs in the absence (●) or in the presence (▲) of 50 μM melatonin. Values are the mean ± S.D. of five separate experiments performed with different RBC preparations.

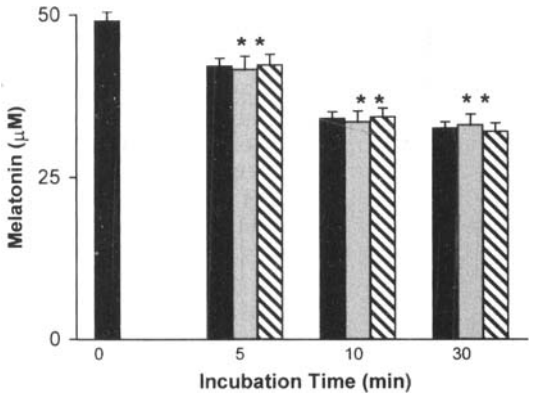

Fig. 8. Melatonin consumption during the cumOOH-induced oxidation of RBCs in the presence of mannitol or DMSA. RBCs (1%) were suspended in hypotonic PBS and the lysed cells were incubated at 37°C with 300 μM cumOOH in the presence of 50 μM melatonin (black bar; control) or 50 μM melatonin plus either 50 mM mannitol (stippled bar) or 50 mM DMSO (dashed bar). Values are the mean ± S.D. of three separate experiments performed with different RBC preparations.

melatonin, consumption of the molecule should be expected to be lower in the presence of excess of high-affinity traps for $^{\bullet}OH$, such as DMSO (rate constant 0.7×10^{10} M/s) [Klein et al., 1981] and mannitol (rate constant 0.27×10^{10} M/s) [Halliwell and Gutteridge, 1989], than in their absence. A 1% RBC suspension, in hypotonic PBS to obtain lysis, underwent oxidation by 300 μM cumOOH in the presence of 50 μM melatonin and in the absence or presence of a 1,000 times excess either DMSO or mannitol. The addition of both traps did not affect the time-course of melatonin consumption (Fig. 8). The consumption rate was the same as that measured in the absence of the hydroxyl radical traps, i.e. 0.27×10^{7} M/s, suggesting that, under our experimental conditions, melatonin is not consumed by reaction with $^{\bullet}OH$. As a corollary, these experiments also provide evidence that melatonin consumption during cumOOH-induced oxidation of lysed RBCs is comparable with that measured in intact RBCs (Fig. 2).

Discussion

It is accepted that the destruction of RBCs by oxidative mechanisms is the end result of two closely related processes; namely, the denaturation of Hb and the oxidation of the RBC membrane. CumOOH, an organic compound frequently used as a model oxidant to initiate radical formation in cell systems [Koster and Slee, 1983; Van den Berg et al., 1992], induces a rapid depletion of reduced GSH in RBCs and brings about Hb oxidation [Tornalley et al., 1983; Van den Berg et al., 1992]. As a consequence, radicals are generated that would enhance intracellular and membrane protein oxidation and lipid peroxidation [Yamamoto et al., 1985], finally leading to cell lysis. This paper shows that melatonin protects human erythrocytes against cumOOH-induced oxidative hemolysis, while it is consumed during the oxidation process.

Protective activity of melatonin appears independent of the membrane lipid oxidation, a process which takes place to the same extent in the absence, as well as in the presence, of melatonin. Rather, melatonin delays membrane protein degradation and precipitation of hemin onto the RBC membrane. These findings are consistent with previous reports that radical-stimulated membrane lipid oxidation and protein degradation proceed via independent mechanisms in erythrocytes [Davies and Goldberg, 1987].

Melatonin has been shown to have lipoperoxyl radical-scavenging activity [Marshall et al., 1996; Livrea et al., 1997]. Therefore, it seems puzzling that it does not protect membrane lipids. Other researchers found a reduced lipid peroxidation in membranes of rat erythrocytes treated with H_2O_2 in the presence of melatonin [Chan and Tang, 1996]. A different oxidation pathway by H_2O_2 versus cumOOH [Van den Berg et al., 1992] may account for this discrepancy. Oxyradicals from cumOOH placed in the membrane [Van den Berg et al., 1992] may spread radical chain reactions through the lipid moiety more rapidly than melatonin can scavenge lipoperoxyl radicals. On the other hand, no melatonin was found at the membrane level in our RBC system.

Hemin accumulation in the membrane of erythrocytes causes a rapid destruction of membrane integrity [Liu et al., 1985; Jarolim et al., 1990; Solar et al., 1990; Schmitt et al., 1993]. By preventing hemin release, melatonin may protect the RBC membrane. We observed that cumOOH-induced osmotic fragility was markedly decreased by melatonin.

Compounds, such as GSH [Shviro and Shaklai, 1987] or desferrioxamine [Baysal et al., 1990; Sullivan et al., 1992; Dailly et al., 1998], have been shown to bind hemin, thereby preventing its precipitation onto the RBC membrane and hemolysis. Our data suggest that mechanisms other than hemin chelation consume melatonin during the cumOOH-induced RBC oxidation.

HbO_2 is the primary target of the cumOOH oxidant activity in RBCs. During RBC oxidation, consumption of melatonin proceeds according to a biphasic curve, with an initial rapid decrease for 10 min, followed by a slower consumption for an

additional 20 min. In addition, the indole is not fully consumed, and its amount remains stable after 30 min. Interestingly, melatonin totally abolishes protein carbonyl accumulation and hemin precipitation onto the RBC membrane during the first 10 min of incubation in the presence of cumOOH, i.e. during the time interval in which the indole is actively consumed. Thus, the indole may counteract the oxidation of Hb, thereby delaying Hb denaturation. Since accumulation of hemin proceeds even when melatonin consumption is no longer observed, it seems conceivable to hypothesize that melatonin protects Hb from denaturation.

The reactivity of melatonin with the highly toxic hydroxyl radical has been repeatedly reported in in vitro studies [Tan et al., 1993; Chen et al., 1995; Sewerynek et al., 1995a,b; Matuszak et al., 1997; Stasica et al., 1998], as well as in vivo [Vijayalaxmi et al., 1995; Li et al., 1997] studies. Due to the high reactivity of the hydroxyl radical, the presence of melatonin may produce substantial protective effects against a variety of cell substrates. Fenton chemistry may produce hydroxyl radicals in our model system [Gutteridge, 1986; Halliwell and Gutteridge, 1989]. However, the experiments carried out in the presence of excess hydroxyl trapping agents, such as mannitol or DMSO, appear to rule out melatonin being consumed in reaction with $^{\bullet}OH$. Protection of protein, but not of lipids or GSH, also supports the view that melatonin is not acting as a preventive antioxidant in our system. Similar reasons may rule out the reaction of melatonin with alkoxyl- or peroxyl-radicals from cumOOH. Possibly, novel hitherto unknown interactions allow melatonin to protect human RBCs from hydroperoxide-induced hemolysis. The mechanism by which melatonin would interfere with cumOOH-induced oxidation of HbO_2 is currently under study in our laboratory (manuscript in preparation). Experiments in which oxidation products from HbO_2 were measured during the cumOOH-induced oxidation of human RBCs and studies of oxidation of *meta*-myoglobin by H_2O_2 in homogeneous solution lead to the conclusion that melatonin can scavenge high-valence-state iron Hb radicals. Electron paramagnetic resonance (EPR) studies provided evidence that such complexes are involved in the oxidation of Hb by hydroperoxides [Giulivi and Cadenas, 1998].

When erythrocytes underwent oxidation by 300 μM cumOOH in the presence of 50 μM melatonin, we observed that only 35% of the indole was consumed. This does not appear to be the consequence of membrane rate-limiting restraints or of a varied membrane permeability during RBC oxidation, but rather of the cumOOH-induced oxidative reactions that melatonin is involved in. Indeed, we observed a comparable consumption of melatonin when lysed instead of intact cells were used. In addition, the increase of the prooxidant in the assay from 300 to 450 μM resulted in twice as much consumption of melatonin. These observations appear particularly suggestive when compared with the results from equilibrium partition studies. Indeed, when a 1% RBC suspension was incubated in the presence of 50 μM melatonin, no melatonin was found in the RBC membrane and only 15% was localized in the cell cytosol. Therefore, the entry of melatonin is strongly favoured when erythrocytes are under an oxidative stress. In other words, oxidative reactions consuming melatonin inside RBCs further promote the entry of melatonin into the cells.

A real involvement of melatonin in the antioxidant protection of cells under physiological conditions has been questioned, both in consideration of the small amounts of circulating melatonin, and of the nature of the radicals generated in cells [Menendez-Pelaez et al., 1993; Marshall et al., 1996]. Erythrocytes may differ from other cells in this respect. In contrast to other cells, erythrocytes have been shown to be capable of an effective uptake and may accumulate melatonin intracellularly [Pablos et al., 1995]. That radical processes involving Hb "draw" even higher amounts of melatonin, and that melatonin inhibits release of hemin may suggest that it could play a significant role in protecting these cells from oxidative stress and damage. Moreover, hemolytic anemia is common in hemoglobinopathies. Putative protective effects of melatonin should be explored in pathological RBCs, in which precipitation of unstable Hb, radical formation, and precipitation of Heinz bodies onto the membrane promote oxidative modifications, leading to cell lysis.

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