Melatonin precludes cytoskeletal collapse caused by hydrogen peroxide: participation of protein kinase C

Gloria Benítez-King†, Leonardo Ortiz-López & Graciela Jiménez-Rubio

†Author for correspondence
Instituto Nacional de Psiquiatría, Neurofarmacología, SIC., Calz. México-Xochimilco No 101, Col. San Lorenzo Huipulco., México, D.F, CP 14370, México
Tel.: +52 555 573 2437
Fax: +52 555 513 3722
bekin@imp.edu.mx

Background: Oxidative stress is a hallmark of aging and most neurodegenerative diseases such as Alzheimers, Parkinsons, and diffuse Lewy body diseases. Hydrogen peroxide (H_2O_2) causes free radical generation and apoptosis similar to that present in neurogenerative diseases. It has been thought that H_2O_2 disorganizes the cortical actin cytoskeleton and, in brain cell extracts, inhibits actin polymerization. Moreover, microtubule network disruption has also been observed in cortical neurons exposed to high levels of free radicals. Melatonin, the main product secreted by the pineal gland, protects the neuronal cells by its action as a free radical scavenger. In addition, this antioxidant inhibits apoptosis and induces microtubule and microfilament reorganization as well as neurite formation in neuronal cultured cells. **Objective**: In this work, we characterized the effects of H_2O_2 on neurite structure and studied the effects of melatonin on cytoskeletal organization in N1E-115 cells damaged by H_2O_2. **Methods**: Melatonin treatment and H_2O_2 effects on neurite cytoskeletal arrangements were evaluated by measuring both the major axis cell length or by simultaneous immunofluorescence staining of microtubules and microfilaments. Free radical levels and cell death were also quantified by measuring lipid peroxides and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method, respectively. **Results**: The results demonstrated that H_2O_2 causes loss of neurites and a cytoskeletal retraction toward the perinuclear region. Melatonin precluded microfilament and microtubule collapse in N1E-115 cells, as well as the increased lipid peroxidation and apoptosis caused by H_2O_2 and restores neurite formation, microtubule enlargement and microfilament organization in microspikes and growth cones in cells damaged with H_2O_2. The results presented here support the idea that an intracellular melatonin mechanism, not mediated by melatonin membrane receptors participates in the mechanisms by which melatonin precludes cytoskeletal damage caused by free radicals. Data show that protein kinase C participates in this mechanism. The protein kinase C agonist, phorbol 12-myristate 13-acetate, caused cytoskeletal reorganization in the presence of H_2O_2, while the protein kinase C inhibitor, bisindolylmaleimide, blocked neurite formation and microfilament reorganization elicited by melatonin. In addition, the calmodulin antagonist, ophiobolin was not capable of protecting cells against the damage caused by H_2O_2. However, phorbol 12-myristate 13-acetate and ophiobolin resembled the melatonin effects in cells treated with H_2O_2. A cytoskeleton organized in neurites and a network all over the cytoplasm was observed. In contrast, the melatonin receptor antagonist did not abolish the protective effects of melatonin against the damage caused by H_2O_2. **Conclusions**: The results presented here support the fact that melatonin may be useful in the treatment of neurodegenerative diseases by restoring microspikes, lamellipodia, growth cones and microtubule enlargement to form neurites that will eventually re-establish synaptic connectivity and an improvement of cognition.

Keywords: cytoskeleton, free radicals, hydrogen peroxide, melatonin, N1E-115 cells, neurodegenerative diseases

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Oxidative stress is a hallmark of aging and most neurodegenerative diseases such as Alzheimers, Parkinsons and diffuse Lewy body diseases [1–2]. It is known that oxygen-reactive species effect proteins, lipids and nucleic acids causing tissue damage and apoptosis [1–2]. Moreover, free radicals produce alterations in cytoskeletal organization, neurite damage and neuronal loss [3,4]. In addition, evidence has suggested that dementias are cytoskeletal disorders that involve loss of axons and dendrites of neurons in the CNS and, consequently, disruption of synaptic connectivity [5].

Hydrogen peroxide (H_2O_2) causes free radical generation and neuronal apoptosis [6]. This compound generates the highly oxygen-reactive

hydroxyl radical that can cross the lipid bilayer and has a significant intracellular diffusion [7]. In leukocytes, H_2O_2 disorganizes the cortical actin cytoskeleton. *In vitro*, this compound inhibits actin polymerization, causes disassembly of microfilaments and affects the interaction between oxidant-stressed actin and DNase I [8]. Microtubule network disruption and neuronal cell death have also been observed in cortical neurons exposed to high levels of free radicals [4]. Moreover, cortical neurons incubated with H_2O_2 showed modifications of β-tubulin or microtubule-associated protein-2 (MAP2) distribution and an abnormal microtubule organization [9]. Recently, it has been shown that H_2O_2 inhibits cytoskeletal dependent processes such as dopamine release in the substantia nigra pars compacta of guinea pigs' brain [10].

Melatonin, the main hormone secreted by the pineal gland, acts as a free radical scavenger, neutralizing, among others, hydroxyl and peroxy radicals, preventing lipid membrane peroxidation and apoptosis [11]. In particular, melatonin prevents free radical-mediated lipid peroxidation induced by H_2O_2 in the brain, total spinal cord, optic nerve and spinal cord white matter, as well as in rat brain homogenates [12–13]. The indole also protects DNA damage induced by H_2O_2 in U-937 cells [14]. Recently, we described in N1E-115 cells that melatonin prevents microtubule disruption, lipid peroxidation and apoptosis induced by okadaic acid, a phosphatase 1 and 2A inhibitor that causes the generation of free radicals [15–16]. In addition, melatonin precludes cytoskeletal collapse produced by H_2O_2 in N1E-115 cells [5]. Direct assessment of melatonin effects on cytoskeletal organization in N1E-115 cells indicates that the indole causes microtubule, microfilament and intermediate filament reorganization as well as neurite formation [17–18]. Melatonin antagonism to Ca^{2+}/calmodulin (CaM) participates in the mechanism by which the indole elicits increased tubulin polymerization, enlargement of microtubules and neurite formation [19]. In addition, selective activation of the α isoform of protein kinase (PK) C by melatonin has been shown to participate in vimentin intermediate filament rearrangements and neurite outgrowth elicited by this indole [20]. Furthermore it has been suggested that stimulation of mt_1 melatonin receptors in N1E-115 cells participate in neurite formation elicited by melatonin [21]. Recently, we showed that melatonin causes increased organization of microfilaments in stress fibers in Madin-Darby canine kidney (MDCK) cells [22].

In this work we characterized the effects of H_2O_2 on neurite structure and the effects of melatonin on cytoskeletal organization in N1E-115 cells incubated with H_2O_2. Melatonin treatment and H_2O_2 effects on neurite cytoskeletal arrangements were evaluated by measuring both the major axis cell length or by simultaneous immunofluorescence staining of microtubules and microfilaments. Lipid peroxidation levels and apoptosis were also quantified. Participation of melatonin mt_1 receptors, PKC and CaM on the protective effects of melatonin against the damage caused by H_2O_2 was determined by selective blockage of these molecules with specific antagonists. The results showed that H_2O_2 causes loss of neurites and a cytoskeletal retraction toward the perinuclear region. Melatonin prevented microfilament and microtubule collapse in N1E-115 cells as well as increased lipid peroxidation and apoptosis caused by H_2O_2. Our findings also indicate that melatonin restores neurite formation, microtubule enlargement and microfilament organization in microspikes and growth cones in cells cultured with H_2O_2. While, the PKC agonist caused cytoskeletal reorganization in the presence of H_2O_2, the PKC inhibitor, bisindolylmaleimide, blocked neurite formation and cytoskeletal reorganization elicited by melatonin. In addition, the CaM antagonist, ophiobolin, was not capable of protecting the cells against the damage caused by H_2O_2. However, phorbol 12-myristate 13-acetate (PMA) and ophiobolin resembled the melatonin effects in cells treated with H_2O_2 and a cytoskeleton organized in neurites and a network all over the cytoplasm was observed. In contrast, the melatonin receptor antagonist did not abolish the protective effects of melatonin against the damage caused by H_2O_2. Our data suggest that melatonin can be a potential therapeutic agent in the treatment of neurodegenerative diseases through prevention of the cytoskeletal damage caused by free radicals and by restoration of cytoskeletal organization and neurite formation.

Materials & methods

Cell culture

Murine neuroblastoma N1E-115 cells (10–90 passage) were cultured in Dulbecco's modified Minimum Eagle's Medium (DMEM) supplemented with 10% bovine serum, 5% horse serum,

100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.025 M 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES). Cells were rinsed twice with phosphate buffered saline (PBS) (0.128 M NaCl; 0.010 M Na_2HPO_4; 0.005 M KH_2PO_4; pH: 7.2), detached and replated on glass coverslips (18 × 18 mm) or in petri dishes. In both cases, cells were replated at low density (20,000 cells/cm^2) as described [18] and cultured for 2 days prior to the experiments.

Light microscopy

In order to characterize optimal H_2O_2 concentration to cause cytoskeletal changes, N1E-115 cells were plated on glass cover slips (18 × 18 mm), and incubated with various H_2O_2 concentrations (10^{-7} or 10^{-5} M, 10^{-4}) for 1 h. After incubation, cells were washed twice with PBS and fixed for 20 min with 4% formaldehyde at room temperature. Fixed cells were stained with 0.02% Coomassie blue according to [23]. Cover slips were mounted on glass slides in glycerol: PBS, 9:1. Cells were observed with an Olympus optic microscope. Images were obtained with a Hitachi KP-D581 color digital camera. Major cell length axes were measured with the Image Pro Express software from Media Cybernetics. For all treatments, the number of measured cells varied from 200 to 300. The absolute values of cell number were normalized to percentage of the total population counted and then subjected to histogram analysis. Experiments were carried out in duplicate and each experiment was performed three times.

Cell treatments

Melatonin and H_2O_2 were obtained from Sigma Chemical Co. (MO, USA). Melatonin was prepared as described [17] and diluted in DMEM. The effects of melatonin on cytoskeletal changes caused by H_2O_2 were evaluated by adding at different times 100 nM melatonin, the indole physiological cerebrospinal fluid circulating concentration [24]. Melatonin was added:

- 3 h before treatment with 100 µM H_2O_2 for 1 h
- Simultaneously with 100 µM H_2O_2 addition
- After treatment with 100 µM H_2O_2 for 1 h

In all experimental groups, total incubation time lasted 4 h. H_2O_2 was dissolved in the incubation media. Participation of melatonin mt_1 receptors, PKC and CaM on the protective effects of melatonin against the damage caused by H_2O_2 was determined by pre-incubation of N1E-115 cells for 20 min with 10 µM with the melatonin antagonist luzindole (N-acetyl-2-benzyltryptamine) [25], the specific PKC inhibitor, bisindolylmaleimide (GF 109203X) (0.5 µM), [26] or with the CaM antagonist ophiobolin (7.5 µM) followed by 4 h incubation with 100 nM melatonin or 2.5 nM of the PKC agonist PMA and 100 µM H_2O_2. Cells were then processed for immunofluorescence, lipid peroxidation assay and apoptosis determination.

Immunofluorescence

N1E-115 cells plated and incubated on glass cover slips were treated as described above and triple stained by immunofluorescence. Briefly, cells were fixed for 15 min at room temperature in 3.7% formaldehyde in PBS and treated with 1 M glycine in PBS for 15 min. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 30 min. Nonspecific sites were blocked with 1% BSA over 30 min. Microtubules and microfilaments were observed by staining of tubulin and actin, respectively. Permeabilized cells were triple-stained with a monoclonal mouse anti-tubulin-β antibody (Zymed Laboratories Inc. CA, USA) and rhodamine-phalloidin (Rh-P) (Sigma Chemical Co. MO, USA). Preparations were incubated at 37°C for 60 min with the anti-tubulin antibody followed by the secondary antibody for 30 min. Microfilaments were simultaneously stained for 30 min with Rh-P. After cytoskeletal staining, nucleus were stained with 20 ng/ml 4,6-diamidino-2-phenylindolar dihydrochloride (DAPI) (Molecular Probes, OR, USA) for 3 min. Cover slips were mounted in 30 µl of prolong antifade. Images were acquired with a Hitachi KP-D581 color digital camera and processed with image analyzer software from Media Cybernetics. Experiments were carried out in triplicate and at least 20 fields were observed for each of the three experiments.

Lipid peroxidation

N1E-115 cells cultured as described were scraped out with a rubber policeman and homogenized with a Cole Palmer ultrasonicator in ice-cold 20 mM Tris-HCl, buffer, pH 7.4. Homogenates were centrifuged at 13,200 x g for 5 min at 4º C. Supernatants were collected and lipid peroxides were measured as malondialdehyde (MDA), and 4-hydroxyalkenals (4-HDA) [27] using the lipoperoxidation assay kit (Calbiochem, CA, USA) MDA and 4-HDA

were quantified at 586 nm. Protein content in cell homogenates was measured by Lowry's method using BSA as a standard [28]

Apoptosis

Apoptotic cells were counted with the DeadEnd fluorometric TUNEL System kit (Promega Co., Madison, WI) [29]. Cells were observed with an Olympus optic microscope and the images obtained with a Hitachi KP-D581 color digital camera. At least 20 randomized fields were examined and damaged nuclei counted. Experiments were carried out in quadruplicate.

Statistical analysis

Results were expressed as mean + standard error of the mean (SEM) values. Data were analyzed by the Student's t test.

Results

Coomassie blue-stained cytoskeletons of vehicle incubated cells were observed in neurites and as a network distributed throughout the cytoplasm (**Figure 1A**). In contrast, cells incubated with H_2O_2 for 1 h showed loss of neurites, a cytoskeletal retraction toward the perinuclear region and changes in cell morphology from elongated to rounded shape (**Figure 1B**). To quantify cytoskeletal retraction, random cell images were obtained, and the length of the major axis determined. The resultant histograms demonstrated that major axis length measured between 70 and 150 µM (**Figure 1C**) in 90% of the vehicle-incubated cells (**Figure 1C**). Increasing concentrations of H_2O_2 caused a reduction in the major axis cell length (**Figure 1D & 1E**). Maximal cell size reduction was obtained with 100 µM H_2O_2. This concentration caused a decrease in neurite size in 100% of the cells and major axis length measured from 10 to 50 µM (**Figure 1F**).

In order to examine whether or not melatonin was capable of blocking the cytoskeletal retraction caused by H_2O_2, N1E-115 cells were incubated with the melatonin physiologic cerebrospinal fluid circulating concentration (10^{-7} M). The indole was added before, together or after H_2O_2 treatment. As shown in **Figure 2A**, in N1E-115 vehicle-incubated cells, microtubules were distributed as a network following the neurite profile, while microfilaments organized in microspikes and growth cones were observed in some cells. H_2O_2-incubated cells showed a collapse of microtubules and microfilaments around the nucleus and cytoskeletal organization in neurites, microspikes and growth cones was lost (**Figure 2B**). Melatonin prevented microtubule and microfilament disorganization when cultures were treated with this indole 3 h before, or at the same time as H_2O_2 treatment (**Figures 2D&E**). Microtubules were distributed in neurites and in some cells were observed as a spread network extending all over the cytoplasm with their distribution pattern similar to vehicle-incubated cells (**Figure 2A**). Nevertheless, cytoskeletal disorganization was established after 1 h H_2O_2 treatment, melatonin was able to restore microtubule organization and neurites and the microtubule network was observed similar to tubulin staining in the vehicle-incubated cells (**Figure 2F**). Thin neurites were observed in the cells cultured solely with melatonin (**Figure 2C**). Microfilament organization in microspikes and growth cones was preserved in cells incubated with melatonin before, at the same time or restored after H_2O_2 treatment (**Figures 2D– F**).

To quantify the protective effect of melatonin against the cytoskeletal damage caused by H_2O_2, rounded cells having a major length cell axis of 10 to 50 µM were counted. The resultant histograms were obtained by normalizing the number of rounded cells to the total cell population (100%). Melatonin significantly reduces the number of cells with a disorganized cytoskeleton, as shown in **Figure 3**. The vehicle-incubated cells showed a small proportion of rounded cells (7%) (**Figure 3**). While H_2O_2, augmented the rounded cells by 76%. A small decrease in the number of rounded cells was observed in the cells solely incubated with melatonin when compared with the vehicle incubated cells (**Figure 3**). In contrast, 70% reduction of rounded cells was obtained when the cells were treated with melatonin for 3 h before the H_2O_2 addition (**Figure 3**). Melatonin added simultaneously with H_2O_2 caused a 72% decrease in the number of cells with a collapsed cytoskeleton when compared with H_2O_2-incubated cells (**Figure 3**). The effect of H_2O_2 was reversed by 75% when N1E-115 cells were treated with melatonin 3 h after H_2O_2 (**Figure 3**). These results indicate that melatonin effectively protects cytoskeletal organization against the damage caused by 100 µM H_2O_2 and confirms that the physiologic cerebrospinal fluid melatonin concentration prevents H_2O_2-induced cytoskeletal collapse.

To determine whether both cytoskeletal damage and reorganization correlated with free radical levels we measured lipid peroxidation levels in cells treated with H_2O_2, with or without; melatonin. H_2O_2 increased lipoperoxide

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Affiliations

Gloria Benítez-King
Instituto Nacional de Psiquiatría, Neurofarmacología, SIC., Calz. México-Xochimilco No 101, Col. San Lorenzo Huipulco., México, D.F, CP 14370, México
Tel.: +52 555 573 2437
Fax: +52 555 513 3722
bekin@imp.edu.mx

Leonardo Ortiz-López
Instituto Nacional de Psiquiatría, Neurofarmacología, SIC., Calz. México-Xochimilco No 101, Col. San Lorenzo Huipulco. México, D.F., CP 14370, México
Tel.: +52 555 655 2811 ext 119
Fax: +52 555 513 3722
leonardoortz@yahoo.com

Graciela Jiménez
Instituto Nacional de Psiquiatría, Neurofarmacología, SIC., Calz. México-Xochimilco No 101, Col. San Lorenzo Huipulco., México, D.F., CP 14370, México
Tel.: +52 555 628 2811 ext 119
Fax: +52 555 513 3722
graylin@imp.edu.mx