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Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells

Abstract: The role of melatonin in the mediation of apoptotic events has recently gained attention, especially after recent studies have reported that melatonin exerts antiapoptotic actions in normal cells but may activate proapoptotic pathways in some tumor cells. Here, we have evaluated the effect of melatonin on apoptosis in the human leukemia cell line HL-60. Melatonin treatment (1 mM) induced a significant increase in caspase-3 and -9 activities. The effect of melatonin on the activation of caspases was time dependent, reaching a maximum after 12 hr of stimulation, and then decreasing to a minimum after 72 hr. Treatment with melatonin also evoked mitochondrial membrane depolarization and permeability transition pore induction, which caused loss of mitochondrial staining by calcein, and increased cell death by apoptosis/necrosis as demonstrated by propidium iodide positive-staining of cells after 72 hr of stimulation. In addition, the exposure of cells to melatonin resulted in an activation and association of the proapoptotic proteins Bax and Bid, as well as promoting detectable increases in the expression of both proteins. We conclude that melatonin has proapoptotic and/or oncostatic effects in the human myeloid cell line HL-60.

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Introduction

One of the earliest and most consistently observed features of apoptosis is the activation of a series of cytosolic cysteine proteases, the caspases, which cleave multiple protein substrates *en mase*, leading to the loss of cellular structure and function, and ultimately resulting in cell death [1]. The caspase family consists of at least 14 enzymes in mammalian cells, of which 11 have been identified to be expressed in human cells. In particular, caspases-3, -8, and -9 play a relevant role in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in the Fas/CD95 pathway, and caspase-3, more downstream, is an executioner caspase-activated by multiple pathways [2].

Traditionally, two general pathways to apoptosis have been described. One pathway is the so-called extrinsic pathway initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor, such as Fas [3]. The second pathway is the intrinsic pathway, which is mediated by mitochondrial alterations. In response to apoptotic stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm [4]. One of these well-characterized proteins is cytochrome *c*, which mediates the activation of caspase-9 [5], triggering, in turn, a cascade of caspase activations, including caspase-3, which promotes cellular apoptosis.

Melatonin, the main secretory product of the pineal gland, has many effects on a wide range of physiological functions [6–8]. In the last decade a novel effect of melatonin on apoptosis was reported, where melatonin has been shown to protect normal cells from apoptosis [9, 10]; conversely, in various cancer cells it was found to induce apoptosis [for review see 11]. For example, melatonin reportedly promotes apoptotic cell death in several cancer cells including human myeloid HL-60 cells [12], B-lymphoma cells [13], HT-29 human colorectal cancer cells [14, 15], and rat pituitary prolactin-secreting tumor cells [16]. There is still controversy, however, concerning the proapoptotic effects of melatonin, since some reports claim that melatonin prevented apoptosis of tumor cells both in vitro [17–19] and in vivo [20, 21].

The mechanisms by which melatonin affects apoptosis have not been fully determined, although different modes of action have been proposed [22]. Increasing evidence for a melatonin–mitochondrial relationship includes the apoptotic properties of melatonin through its interaction with the mitochondrial permeability transition pore [10, 23, 24]. Melatonin is a highly lipophilic molecule that easily crosses cell membranes to reach intracellular organelles including mitochondria.

In this study, we have investigated the time-dependent effects and mechanisms of melatonin action on the mitochondrial-mediated apoptosis and cell proliferation of human myeloid HL-60 cells in culture analyzing caspase-3 and -9 activation, mitochondrial depolarization, induction

of the mitochondrial permeability transition pore, and activation of the proapoptotic proteins Bax and Bid.

Materials and methods

Materials

HL-60 15-12 cell line (ECACC No 88120805) is a variant of HL-60 which is differentiating towards either neutrophils or monocytes, it was purchased from The European Collection of Cell Cultures (ECACC) (Dorset, UK). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from HyClone (Aalst, Belgium). L-Glutamine, RPMI 1640 medium were obtained from Cambrex (Verviers, Belgium). Dimethil sulfoxide (DMSO), carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), cyclosporine A (CsA), staurosporine, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), dithiothreitol (DTT) and anti-Bax monoclonal antibody (Clone 6A7) were obtained from Sigma (Madrid, Spain). *N*-acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (AC-LEHD-AMC) was from Bachem (Bubendorf, Switzerland). Calcein-AM and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were obtained from Molecular Probes (Barcelona, Spain). Propidium iodide (PI) and annexin-V-FITC from Immunostep (Salamanca, Spain). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Santa Cruz (Santa Cruz, CA, USA). Protein A-agarose and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody was from Upstate Biotechnology Inc. (Madrid, Spain). Anti-Bid antibody was from Cell Signaling (Barcelona, Spain). Hyperfilm ECL was from Amersham (Arlington Heights, IL, USA). All other reagents were of analytical grade.

Cell culture

Human promyelocytic leukemia HL-60 cells (passages 6–12) were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1.25% DMSO at 37°C under a humidified condition of 95% air and 5% CO_2. Cells were routinely plated at a density of 3×10^5 cells/mL into fresh flasks and the viability was $>95\%$ in all experiments as assayed by the trypan-blue exclusion method.

Assay for caspase activity

To determine caspase-3 and -9 activity, stimulated or resting cells were sonicated and cell lysates were incubated with 2 mL of substrate solution (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 μM of caspase substrate) for 1 hr at 37°C as previously described [25]. The activities of caspase-3 and -9 were calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9). Substrate cleavage was measured with a fluorescence spectrophotometer with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHD-FMK, respectively. The data were calculated as fluorescence units/mg protein and presented as fold increase over the pretreatment level (experimental/control).

Mitochondrial membrane potential determination

Cells were incubated with 1 μM JC-1 for 15 min at 37°C as previously described [26]. The cationic dye, JC-1, exhibits potential-dependent accumulation in the mitochondria. It indicates mitochondria depolarization by a decrease in the red to green fluorescence intensity ratio. After incubation with JC-1, the dye was removed; the cells were washed in phosphate buffered saline (PBS). The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, the red signal at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analyzed using a fluorescence spectrophotometer. Data are presented as emission ratios (590/535). Melatonin-induced changes in mitochondrial membrane potential were quantified as the integral of the decrease in JC-1 fluorescence ratio for 1.5 min after the addition of melatonin and presented as percentage of the effect evoked by 1 μM FCCP.

Calcein and Cl_2Co co-loading

Cells were loaded with 1 μM calcein-AM in the presence of 1 mM Cl_2Co during 15 min at 37°C and later they were washed using always the same RPMI medium where the cells were grown. Mitochondrial calcein fluorescence was measured with a fluorescence spectrophotometer with excitation wavelength of 488 nm and emission at 515 nm [27]. Data are expressed as fractional changes of emitted fluorescence (F/F_0).

Annexin V staining

Cells were harvested through and washed twice with PBS and centrifuged at 500 g for 5 min, then the supernatant was discarded and the pellet was resuspended in 95 μL annexin V-binding buffer at a density of 10^5–10^6 cells/mL containing annexin-V-FITC. Cells were analyzed by cytometry (Beckton Dickinson) after addition of propidium iodide (PI). Ten-thousand events were analyzed using the FL-1 and FL-3 detector filters. Each sample was tested three to five times in independent experiments. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin and PI).

Cell cycle analysis

After treatment, cells (approximately 1×10^6) were washed with PBS and fixed in 70% ethanol for 30 min at 4°C. The cells were again rinsed with PBS and resuspended in 500 μL

of PBS containing 2.5 mg/mL PI and 50 mg/mL RNase. The sample were kept in the dark at 4°C for 30 min and analyzed by flow cytometry with excitation at 488 nm and emission at measured at 560–640 nm (FL2 mode) (Beckton Dickinson). Cells undergoing apoptosis stain with PI and exhibit a reduced DNA content with a peak in the hypodiploid region [16]. The percentage of every phase was represented and the percentage of apoptosis was taken as the fraction with hypodiploid DNA content.

Immunoprecipitation and Western blotting

Bax and Bid expression in HL-60 cells was analyzed by Western blotting. Bax activation and association with Bid were determined by immunoprecipitation as previously described [28]. Briefly, HL-60 cell suspensions (4×10^6 cells/mL) were stimulated, as indicated, and lysed. Following preparation of the lysate, protein concentration was determined using the Bradford assay and adjusted to a protein concentration of 50 μg/mL with lysis buffer. Bax protein was immunoprecipitated from cell lysates by incubation with 2 μg of anti-Bax antibody (clone 6A7) and 15 μL of protein A-agarose overnight at 4°C on a rocking platform. Proteins were separated by 15% SDS–PAGE and electrophoretically transferred, for 2 hr at 0.8 mA/cm^2, in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs, UK) onto nitrocellulose for subsequent Western blotting. Non-specific protein binding sites of the nitrocellulose membranes were blocked by incubating overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST). Membranes were incubated with the anti-Bax antibody diluted 1:200 in TBST for 2 hr or with the anti-Bid antibody diluted 1:500 overnight. To detect the primary antibodies, blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody diluted 1:5000 or horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody diluted 1:10,000 in TBST, respectively, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry.

Statistical analysis

Data are present as mean ± standard error of mean (S.E.M.) and analysis of statistical significance was performed using Student's *t*-test. To compare the different times of melatonin treatments, statistical significance was calculated by one-way analysis of variance followed by Tukey's multiple comparison tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

To examine the effect of melatonin on caspase activation, HL-60 cells were treated with melatonin for 1–72 hr. As shown in Fig. 1A, treatment of cells with 1 mM melatonin induced a time-dependent biphasic activation of caspase-3. Cell stimulation with melatonin caused a detectable activation of caspase-3 after 1 hr of treatment with a 1.3 ± 0.2-fold increase, and the maximum effect was obtained after

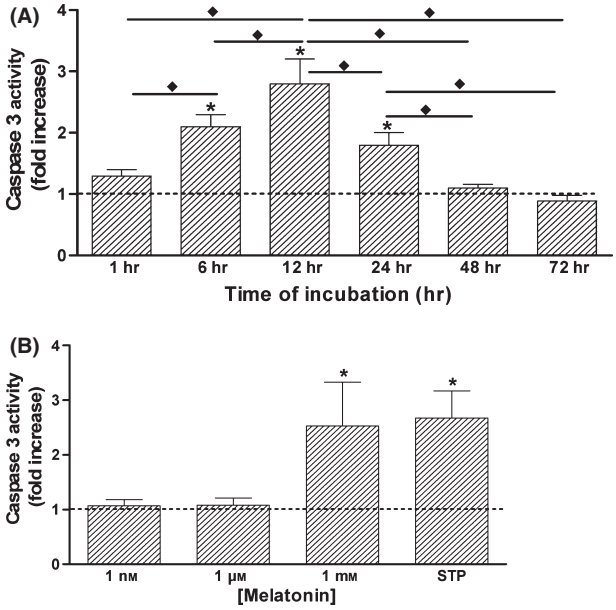

Fig. 1. Time course and concentration dependence of melatonin-induced activation of caspase-3. HL-60 cells were stimulated for various durations (1, 6, 12, 24, 48 and 72 hr) with 1 mM melatonin (A) or for 12 hr with increasing concentration of melatonin (1 nM, 1 μM and 1 mM) (B). The effect of 1 μg/mL staurosporine (STP) treatment for 2 hr on activation of caspase-3 is included in B. Caspase-3 activity was estimated as described under Material and methods. Values are presented as mean ± S.E.M. of 5–8 separate experiments and expressed as fold increase over the pretreatment level (experimental/control). *$P < 0.05$, compared to control values. ♦$P < 0.05$.

12 hr of stimulation (2.8 ± 0.4-fold increase, $P < 0.05$). Thereafter, caspase-3 activity decreased, reaching a minimun at 72 hr after treatment (0.9 ± 0.09-fold increase). Activation of caspase-9 by melatonin showed a similar activation/inactivation curve to that of caspase-3, reaching a maximum after 12 hr of stimulation, with a 2.8 ± 0.6-fold increase, then decreasing to a minimum at 48 hr (1.1 ± 0.2-fold increase, Fig. 2A).

The effect of melatonin on caspase-3 and -9 activation was not clearly concentration-dependent, as shown in Figs 1B and 2B. In fact, treatment of melatonin for 12 hr at 1 nM or 1 μM was unable to induce activation of caspase-3 and -9. Only the higher concentration of melatonin used (1 mM) induced significant rises in caspase-3 and -9 activities (2.8 ± 0.4 and 2.8 ± 0.6-fold increase, respectively, $P < 0.05$). A similar effect obtained with 1 mM melatonin was observed when caspase-3 and -9 activation were stimulated by the treatment with staurosporine (a potent inhibitor of survival protein kinases, which is widely used to activate apoptosis [29]), which elicited a significant increase on activity of caspase-3 and -9 of 2.9 ± 0.3 and 2.5 ± 0.08-fold increase, respectively, after 2 hr of stimulation with 1 μg/mL staurosporine (Figs 1B and 2B).

Treatment of HL-60 cells with 1 mM melatonin induced a mitochondrial membrane depolarization as detected by the decrease in the mitochondrial-specific voltage-sensitive dye JC-1 fluorescence ratio (Fig. 3). Treatment with 1 μM FCCP, a mitochondrial uncoupler which collapses the

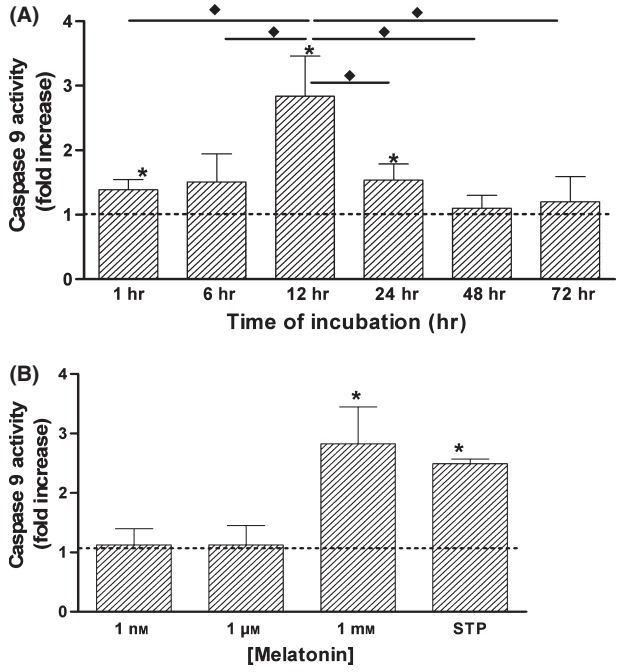

Fig. 2. Time course and concentration dependence of melatonin-induced activation of caspase-9. HL-60 cells were stimulated for various periods of time (1, 6, 12, 24, 48 and 72 hr) with 1 mM melatonin (A) or for 12 hr with increasing concentration of melatonin (1 nM, 1 μM and 1 mM) (B). The effect of 1 μg/mL staurosporine (STP) treatment for 2 hr on activation of caspase-9 is included in (B). Caspase-9 activity was estimated as described under Material and methods. Values are presented as mean ± S.E.M. of 5–8 separate experiments and expressed as fold increase over the pretreatment level (experimental/control). $^{*}P < 0.05$, compared with control values. $^{\blacklozenge}P < 0.05$.

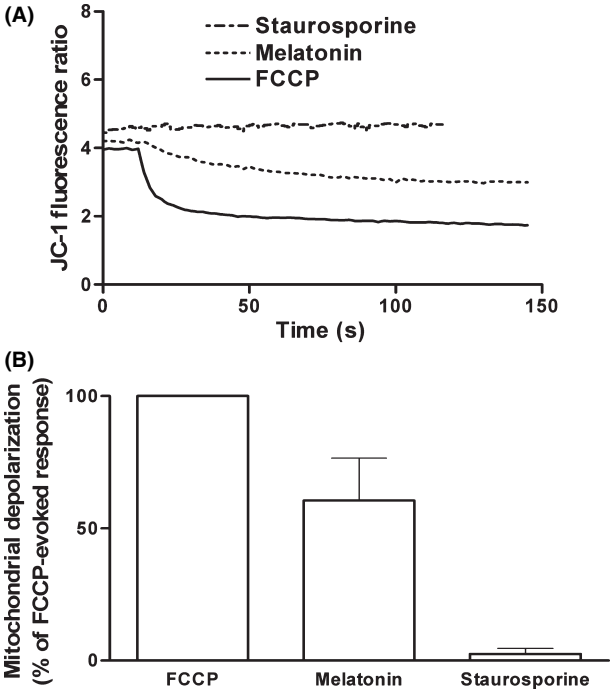

Fig. 3. Mitochondrial membrane depolarization in HL-60 cells. Cells were loaded with JC-1 and then stimulated with 1 μM FCCP, 1 mM melatonin or 1 μg/mL staurosporine, as indicated. (A) Changes in fluorescence were determined as shown under Material and methods. Traces are representative of 4–5 independent experiments. (B) Histogram represents the changes in mitochondrial membrane potential induced by melatonin or staurosporine relative to the effect of FCCP and estimated as described in the Material and methods section. Data are presented as mean ± S.E.M. of 4–5 independent experiments.

mitochondrial membrane potential, induced maximal reduction in JC-1 fluorescence. Depolarization observed in the presence of melatonin was 60 ± 16% of that observed with FCCP (Fig. 3B). However, treatment of cells with 1 μg/mL staurosporine exerted a negligible effect on mitochondrial membrane potential, which confirms that staurosporine-induced apoptosis does not involve mitochondrial depolarization, as previously reported [29].

To test the effect of melatonin on permeability transition pore induction, cells were loaded with calcein-AM in the presence of cobalt chloride to quench fluorescence from all cellular domains except from within mitochondria [27]. Using this protocol, 1 mM melatonin caused a loss of mitochondrial calcein fluorescence, which was completely blocked by pretreatment of cells with the permeability transition pore inhibitor cyclosporine A (50 μM) for 10 min (Fig. 4A). Although this protocol does not distinguish between calcein efflux and cobalt influx, it is consistent with induction of the permeability transition pore. As expected, the treatment with 1 μg/mL staurosporine induced no significant effect on opening of the mitochondrial permeability transition pore (Fig. 4B), suggesting that permeability transition pore is not necessary for the initiation of staurosporine-induced apoptosis.

To evaluate the melatonin effect on cell cycle, cells were stained with PI which has affinity for nucleic acids. An increase in hypodipoloid DNA content was found in melatonin treated cells. Melatonin (1 mM) showed an important Sub-G1 arrest at the expense of the remainder phases; especially S and G2/M were reduced after melatonin treatment compared with the control group (Fig. 5A). Furthermore, we observed a time-dependent rise in the sub-G1 phase with melatonin. In addition, treatment of cells with 1 μg/mL staurosporine for 2 hr shows similar results to those obtained after 12 hr of treatment with melatonin, which consisted in an increase in sub-G1 and a decrease in other phases compared with the control group (Fig. 5A). These results are in agreement with the idea that the progression of cell cycle and control of apoptosis are intimately linked with processes that occur in all multicellular organisms in response to different stimuli [13].

FITC-conjugated annexin V and PI staining was used to detect apoptotic and necrotic changes in melatonin-treated HL-60 cells. When cells double-stained with the dyes were analyzed by flow cytometry, the results revealed that a 9 ± 1.5% of control cells had only annexin V positivity (early apoptotic cells) and 4 ± 1% annexin V/PI positivity (necrotic cells) (Fig. 5B). As also shown in Fig. 5B, 1 mM melatonin induced an increase in the percentage of

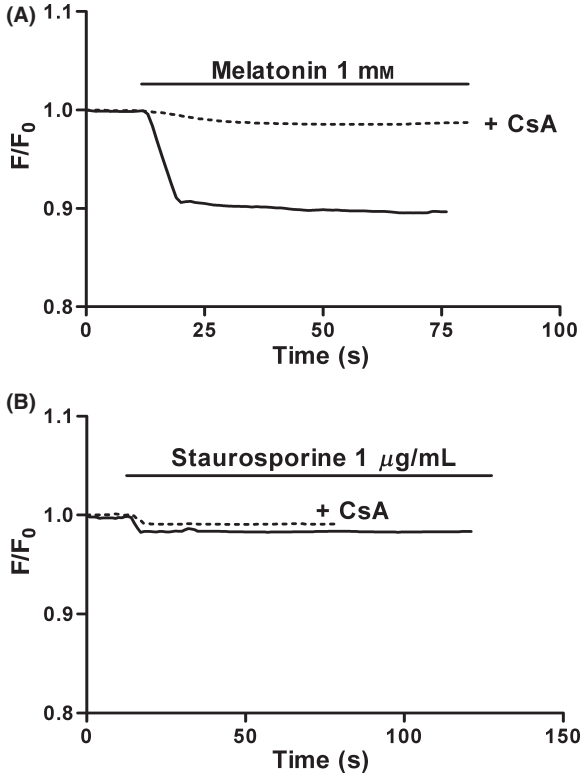

Fig. 4. Melatonin causes a loss of mitochondrial staining by calcein. Calcein-loaded cells in the presence of 1 mM Cl_2Co were preincubated for 10 min with 50 μM cyclosporine A (CsA) or the vehicle (DMSO) and then were stimulated with 1 mM melatonin (A) or 1 μg/mL staurosporine (B). Changes in calcein fluorescence were determined as shown under Material and methods and are expressed as fractional changes of emitted fluorescence (F/F_0). Traces are representative of 5–9 independent experiments.

apoptotic cells. The population of early apoptotic cells increased after 12 or 72 hr treatment with 1 mM melatonin (38 ± 0.2% and 35 ± 1.5%, respectively, Fig. 5B). In addition, the treatment of cells for 12 hr with melatonin induced a slight increase in the population of necrotic cells (cells stained with both annexin V and PI) as compared with the control group (9 ± 0.8% versus 4 ± 1%). However, when the cells were incubated with melatonin for 72 hr the population of necrotic cells was larger compared with the treatment for 12 hr (21 ± 1.6% versus 9 ± 0.8%). For comparison, the effect of 1 μg/mL staurosporine for 2 hr is included in Fig. 5B. Staurosporine treatment elevated the number of apoptotic cells by 24 ± 1.5%, as revealed by annexin V assay, but induced a similar percentage of necrotic cells to that obtained in the control group (7 ± 0.6%).

Melatonin promotes apoptosis in several tumor models, including HL-60 and HT-29 cells, as well as in pituitary prolactin-secreting tumor and human colon cancer cells [12, 14, 16]. Furthermore, apoptosis leads to loss of mitochondrial membrane integrity that often results in activation of pro-apoptotic proteins, including Bax and Bid [12, 30]. To identify possible intracellular pro-apoptotic targets of melatonin during the promotion of apoptosis in HL-60 cells, we investigated the expression and activation of Bax and Bid after treatment with melatonin. We analyzed Bid and Bax activation and association by co-immunoprecipitation using a mouse monoclonal anti-Bax antibody (clone 6A7) that recognizes a specific region that is close to the Bax dimerization domain which is only exposed once Bax is activated in its monomeric conformation [31]. As shown in Fig. 6A, incubation of HL-60 cells with either 1 mM of melatonin for 12 hr and 72 hr or with 1 μg/mL of staurosporine for 2 hr enhanced Bax activity, which reached a 1.17 ± 0.07, 1.14 ± 0.05 and 1.23 ± 0.13-fold increase, respectively ($P < 0.05$; n = 6). Furthermore, we found that treatment with 1 mM melatonin produced a significant and time-dependent rise in the association between active Bax and Bid, which reached a 1.84 ± 0.26-fold increase over the pretreatment level after 12 hr of treatment (Fig. 6B, line 2; $P < 0.001$, n = 6) and a 2.0 ± 0.39-fold increase after 72 hr (Fig. 6B, line 3; $P < 0.001$, n = 5). Additionally, incubation with 1 μg/mL staurosporine for 2 hr significantly enhanced Bax and Bid association by a 2.21 ± 0.37-fold increase (Fig. 6B, line 4; $P < 0.001$, n = 4).

Melatonin also induced a biphasic and time-dependent elevation in Bax and Bid expression as analyzed by Western blotting. We found maximal values of Bax and Bid expression after 12 hr, with 1.21 ± 0.09 and 1.14 ± 0.01-fold increase, respectively (Fig. 6, $P < 0.01$, n = 4–6). Thereafter, Bid expression fell and 72 hr after the addition of melatonin the amount of Bid detected was similar to that in controls. In contrast, Bax expression remained elevated for at least 72 hr (Fig. 6A).

Discussion

The leukemia HL-60 cell line is a useful model to study cell growth of leukemia cells. Melatonin is an indoleamine that has been demonstrated to exert antiproliferative activity in numerous cell types [32–34]; however, studies on leukemia cells are rare. Since time-dependent effects on anti-proliferative and pro-apoptotic properties of melatonin have not been described previously, this study was designed to determine the time-dependent effect of melatonin on mitochondrial apoptosis/necrosis in the HL-60 tumor cell line. Our results demonstrate that melatonin induces apoptotic events in HL-60 cells, which are primarily of mitochondrial origin; signs of this included mitochondrial membrane depolarization and permeability transition pore induction, as well as activation of caspase-9 and -3.

Caspase-9 and -3 are major mediators of apoptotic cell death and their activation is widely considered an apoptotic marker. Here, we show that stimulation of HL-60 cells with millimolar concentrations of melatonin increased caspase-9 and -3 activities, while micromolar concentrations proved to be ineffective. Our results are in agreement with previous studies reporting activation of executioner caspase-3 by melatonin in the same cell line [12], which is activated by either extrinsic or mitochondrial-mediated (intrinsic) pathway [2]. Additionally, our results also demonstrate a time-dependent activation/deactivation of initiator caspase-9 induced by treatment with melatonin, reaching a maximal effect after 12 hr of stimulation. Thereafter, caspase activity decreased. Our results differ partially with those found

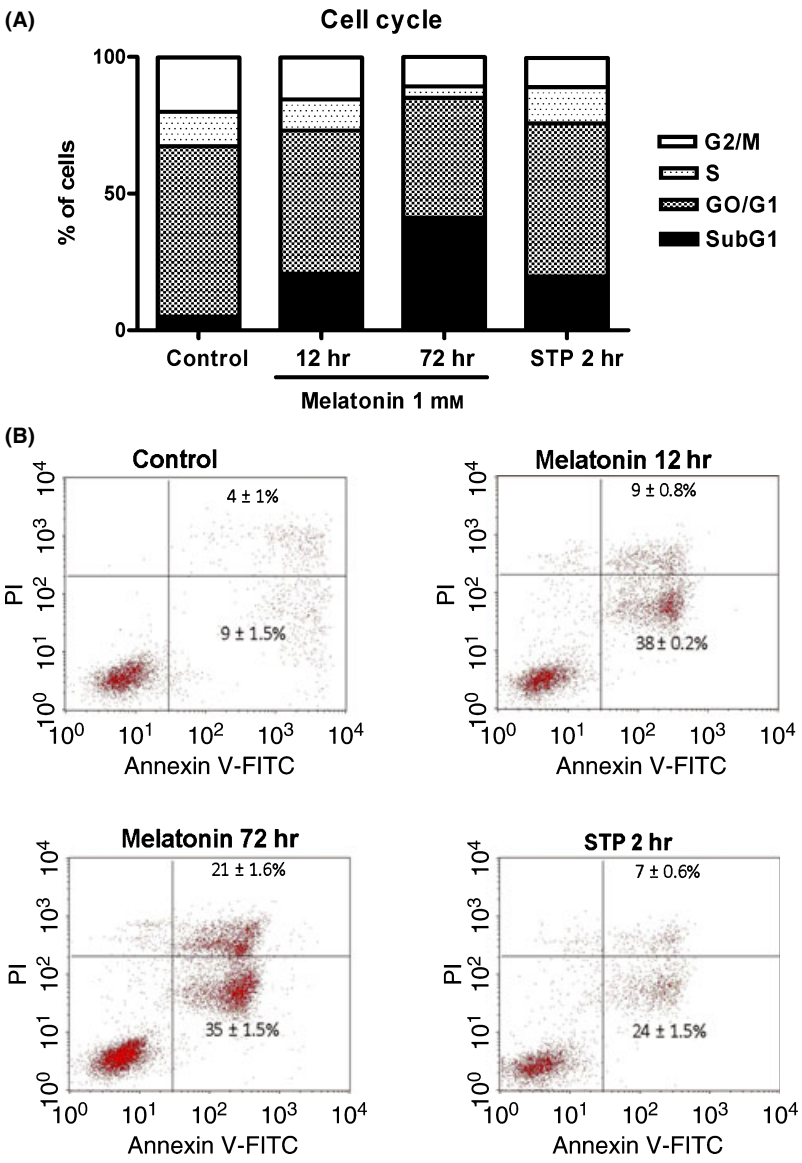

Fig. 5. Effect of melatonin on apoptosis induction in HL-60 cells. Cells were incubated in absence (control) or presence melatonin (1 mM) or staurosporine (STP) (1 μg/mL) for the time period indicated. (A) The cell cycle phase distribution was determined by flow cytometry analysis using ethanol-fixed, propidium iodide-stained cells. The results are represented as the distribution of the cell-cycle phase's percentage and are representative of 3 independent experiments. (B) After treatments, cells were stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Data are representative of 2–3 independent experiments. The percentages are mean ± S.E.M. of 2–3 separate experiments.

previously by Rubio et al. [12], who observed the highest melatonin-induced caspase-3 activity after 72 hr of incubation, time period in which we found the minimum caspase-3 and -9 activities. This apparent controversy could be possibly due to the different methodological procedures employed to determine caspase activity. In our study, we used a spectrofluorimetric method to determine caspase activation, whereas Rubio et al. [12] used a colorimetric enzymatic assay. Furthermore, we cannot reject other reasons related to the cell culture conditions and/or passage number as a possible explanation for differences between our results and those of Rubio et al. [12]. Several studies using other tumor cell types have also reported that melatonin may increase caspase activation [15, 35, 36]. In addition, the findings demonstrate that the caspase activation evoked by melatonin is accompanied by mitochondrial depolarization and induction of mitochondrial permeability transition pore, which strongly suggests involvement of the mitochondrial-mediated (intrinsic) pathway of apoptosis. In fact, the effect of melatonin on mitochondrial depolarization was similar to that obtained when the cells were treated with the mitochondrial uncoupler FCCP. Additionally, melatonin was able to provoke the induction of the mitochondrial permeability transition pore as detected by the reduction in the mitochondrial calcein fluorescence, which was blocked by cyclosporine A, an inhibitor of the induction of the permeability transition pore. These results are in agreement with previous findings which showed a release of pro-apoptotic agents from mitochondria induced by melatonin in tumor cells [13, 36]. It is also worth noting that staurosporine-induced caspase activation is not due to a mitochondrial depolarization and the subsequent opening of permeability transition pore, as it has previously shown [29].

Several studies have reported that melatonin may reduce cell proliferation rate [12, 37] by mechanisms that involve cell death by apoptosis [13, 18]. In this study, we showed that the effects of melatonin on caspase activation are

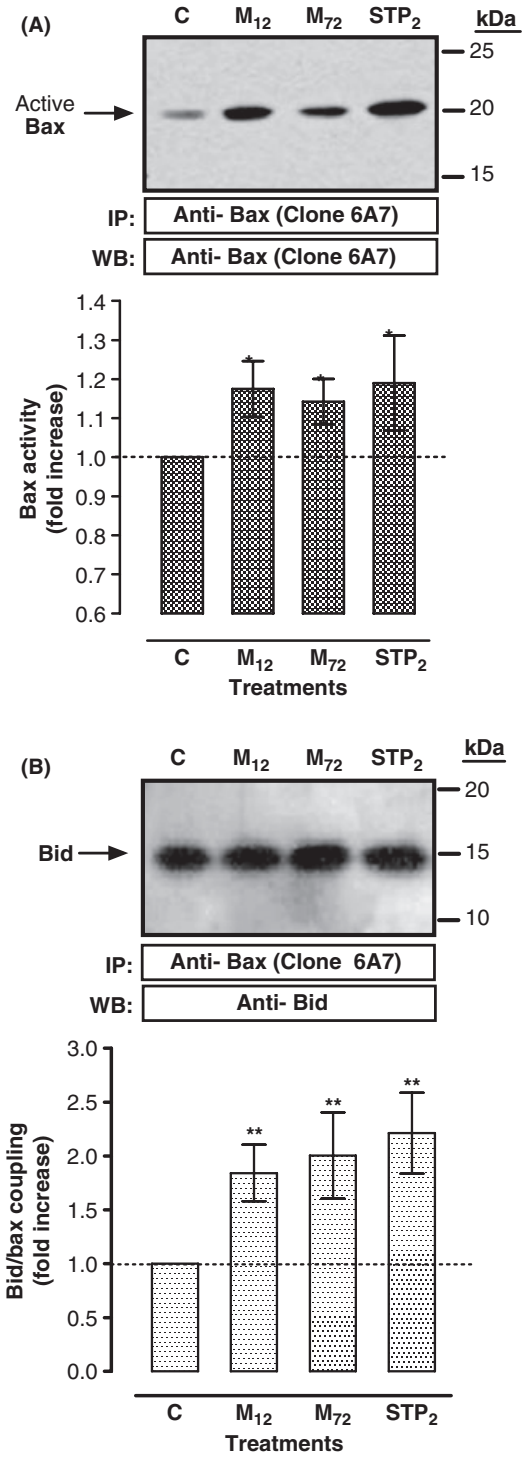

Fig. 6. Effects of melatonin on Bax activation and association with Bid. HL-60 cells were treated with 1 mM melatonin for 12–72 hr (M_{12}–M_{72}) or for 2 hr with 1 μg/mL staurosporine (STP_2), and then lysed. Samples were immunoprecipitated with anti-Bax antibody (clone 6A7). Immunoprecipitates were analyzed by Western blotting using anti-Bax antibody (A) or anti-Bid antibody (B) as described under Material and methods. Histograms show Bax activity (A) or the Bax/Bid association (B) expressed as fold-increase over the pretreatment level (experimental/control). Data are mean ± S.E.M. of 4–6 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with control (C) values.

associated with alterations in the cell cycle progression because melatonin caused Sub-G1 arrest at the expense of S and G2/M phases. Moreover, we have observed a time-dependent rise in the sub-G1 phase induced by melatonin. Our results are consistent with the idea that the progression of cell cycle and control of apoptosis are intimately linked with processes that occur in all multicellular organisms in response to different stimuli [13]. Additionally, treatment with melatonin proved to be effective in increasing the population of necrotic cells, especially at 72 hr after its administration, as detected by the increase in the number of cells stainable with annexin V and PI; this suggests that melatonin may play an important role as a tumor suppressor and/or chemotherapeutic agent against tumor. These results, taken together with the findings on caspase activation, suggest that treatment with melatonin for 72 hr cause the cells to become necrotic.

During apoptosis, several members of the Bcl-2 family undergo activation. For instance, caspase 8 activation, via stimulation of Fas on the plasma membrane, induces rapid activation of Bid and evokes its translocation to mitochondria as tBid. tBid, with 15 kDa, binds Bax favoring their anchoring to the mitochondrial membrane, a process that is required for the activation of MAP-1 and the release of cytochrome *c* [38–40]. The findings show that melatonin induces the activation and association of Bax and Bid, as well as a detectable rise in the expression of both proteins, in HL-60 cells. Similar results have been previously observed by Rubio et al. [12], which reported upregulation of Bax induced by melatonin treatment for 72 hr. The effects of melatonin in HL-60 cells are not totally in agreement with other anticancer drugs that have been evaluated in this cell model, such as furanodiene, which is only able to alter Bid activity, meanwhile other Bcl-2 family members like Bax or Bcl-2 remain unaltered [41]. The ability of melatonin to activate Bax and promote Bax/Bid association strongly suggests that it is a more efficient oncostatic drug than others reported [41]. Further tests are needed to evaluate other possible targets of melatonin in cancer cells, which might be responsible for the induction of necrosis under longer incubation periods.

In this investigation, we have shown that melatonin causes a time-dependent activation/deactivation of caspase-3 and -9. Our studies also demonstrate that apoptotic events induced by melatonin are mainly of mitochondrial origin, and melatonin is able to increase cell death by apoptosis and/or necrosis. We conclude that melatonin has proapoptotic and/or oncostatic effects on human myeloid cell line HL-60 and it could be a candidate as a chemotherapeutic agent against tumors of this type.

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