Melatonin prevents hydrogen peroxide-induced apoptotic cell death

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Summary
Melatonin (MEL) functions in organisms are diverse. The actions considered in the current work relate to its ability to prevent oxidative stress, i.e. molecular damage produced by reactive oxygen species (ROS). Apoptosis is an active form of cell death that is initiated by a variety of stimuli, some of which inducing ROS increase. Hydrogen peroxide (H2O2) is considered a typical cytotoxic and oxidant agent capable to induce cellular damage through free radical production. In the present work we have investigated its role in the induction of cell death. U937 cells were exposed to 500 µM H2O2 and MEL behaviour, in the presence of this type of oxidative stress, was evaluated by incubating cells with MEL before and after H2O2 exposure. Cytometric and microscopy analyses were utilized and revealed that H2O2 can be considered an apoptotic trigger, if used at proper concentrations. In fact U937 cells, after 500 µM H2O2 exposure, showed chromatin condensation, micronuclei presence, apoptotic bodies and secondary necrosis. MEL, added before H2O2 treatment, significantly reduced apoptotic cell number. On the contrary, MEL incubation after H2O2 induced an increase of apoptotic cell death, and cells in secondary necrosis were detectable. These results indicated that pre-incubation with MEL reduces H2O2-dependent apoptosis in U937 cells, suggesting a capacity of this hormone to interfere with apoptosis induced by ROS increase.

Key words: U937, melatonin, hydrogen peroxide, apoptosis, reactive oxygen species.

Introduction
Melatonin (N-acetyl-5-methoxytryptamine), the hormone produced by the pineal gland, was shown to have significantly broad actions including oncostatic effects (Bejarano et al., 2009), immune system stimulation (Guerrero and Reiter, 2002) and anti-inflammatory functions (Radogna et al., 2009). Subsequently, MEL was identified as a direct free radical scavenger (Reiter and Reiter, 2002) and an indirect antioxidant (Rodriguez et al., 2004). Its function consists in the reduction of oxidative stress, i.e. molecular damage produced by reactive oxygen and nitrogen species (Reiter, 2008). Numerous reports evidenced MEL ability to neutralize free radicals, molecules that have an unpaired electron in their valence orbital, as hydroxyl (•OH) (Li et al., 2002) and oxygen radical (O2••). Melatonin is also a scavenger of hydrogen peroxide (Tan et al., 2000), a non-radical ROS, generated in vivo by several enzyme systems and produced intracellularly by the dismutation of the superoxide anion radical (O2••). In addition, it is known that MEL acts by indirectly stimulating the antioxidative enzymes, that represent a major defence mechanism against free radical damage.

Literature evidences that MEL, through both antioxidant and scavenger functions, plays an important role in preventing apoptosis: its anti-apoptotic activity was described in several systems, including cerebellar neurons (Baydas et al., 2002) and brain astrocytes (Jou et al., 2004), where it was hypothesized to have an antioxidant
role. A recent study demonstrated MEL capacity to prevent UVB-induced apoptosis in U937 cellular line (Luchetti et al., 2006). In the present work, we have evaluated, by means of cytometric and morphological techniques, cell death induced by H$_2$O$_2$ treatment in the same cells, investigating, in particular, MEL function in preventing cell damage induced by ROS increase generated by H$_2$O$_2$ exposure.

**Materials and Methods**

**Cell culture**

U937 human myelomonocytic lymphoma cell line was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 25 mM Hepes pH 7.5, 1% antibiotics and maintained at 37°C in humified air with 5% CO$_2$. Cell viability was assessed by MTT test (Mosmann, 1983).

For the induction of apoptosis, cells (seeded at 1x10$^6$ cells/mL) were exposed to 500 µM H$_2$O$_2$ (Fukamachi et al., 1998) for 3h at 37°C in humidified air with 5% CO$_2$. MEL (Sigma) was first dissolved in absolute ethanol at initial 100 mM concentration and then diluted at final 1 mM concentration in culture medium. 1 mM MEL treatment (Luchetti et al., 2006) was performed both immediately before and after H$_2$O$_2$ treatment; subsequently, the treated cells were post-incubated as described above.

**Flow cytometry (FC)**

Cell death features (early and late apoptotic, as well as necrotic cells) were evaluated using propidium iodide (PI) staining carried out according to manufacturer’s instructions. For FACs analysis 1x10$^6$ cells were collected by centrifugation at 300 g for 5 min at 4°C and washed once in ice-cold PBS. Cells were fixed (and permeabilized) with -20°C cold 70% ethanol overnight at 4°C. Fixed cells were washed in PBS once and resuspended in 1 mL of staining solution (40 µg/mL Propidium Iodide and 100 µg/mL RNase A in PBS). The samples were incubated for 30 min at room temperature in the dark. All samples were analyzed by EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA) with the appropriate software (System II, Beckman Coulter). Data were analyzed and histograms were plotted using winMDI version 2.9 flow cytometry application software (Scripps Research Institute, La Jolla, CA, USA). Values of PI fluorescence were presented as DNA histograms, showing cell distribution into the main cycle phases (G0/M=M1, S=M2, G2-M=M3) and where the possible presence of apoptotic cell death can be recognize by typical sub-G1 peak (M4).

**Light and electron microscopy**

Differently treated U937 pellets were immediately fixed in 2.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer pH 7.3, postfixed in 1% OsO$_4$ in the same buffer, dehydrated with ethanol and embedded in araldite as previously described (Burattini et al., 2009). Semithin sections were stained at 50-60°C with a mixture of 1% methylene blue and 1% toluidine blue in distilled water. Thin sections were collected on nickel grids, stained with uranyl acetate and lead citrate, and observed with a Philips CM10 electron microscope.

**Results**

MTT test (Figure 1) revealed a good cell vitality for control (100%) and MEL alone treated (95.4%) cells, but it decreased to 75% after H$_2$O$_2$. MEL added before H$_2$O$_2$ treatment reduced cell death and viability was 83.9%; on the contrary, MEL added after H$_2$O$_2$ exposure showed a behaviour similar to that with H$_2$O$_2$ alone, and cell vitality was 74%.

Flow cytometry (Figure 2) evidenced that both control (2A) and cells treated with MEL alone (2B) showed only 1.5% and 2.9%, apoptotic/necrotic cells respectively, revealing a good cell viability.

For both conditions a well preserved cell morphology was confirmed by reverse microscope (RM), semithin sections and ultrastructural analyses (Figure 3A, B, C, D; 4A, B).

PI staining showed that H$_2$O$_2$ treatment (Figure 2C) induced apoptosis, quantified as 10.6% of PI / FITC ANX-V positive cells. RM and LM showed a variety of apoptotic cells (Figure 3E, F). TEM observations, after H$_2$O$_2$ treatment, evidenced characteristic apoptotic patterns (Figure 4C), such as chromatin margination towards nuclear periphery with typical cup-shaped patches (Figure 4D). Micronuclei, scattered throughout the cytoplasm and progressively released in...
extracellular space, characterize the late apoptotic stage (Figure 4E).

When cells were treated with 1mM MEL before H$_2$O$_2$, apoptotic cell number was similar to that of the control (Figure 2D). Its percentage appeared, in fact, strongly decreased (2.9%), as confirmed by RM, LM and TEM, that showed a low number of apoptotic cells (Figure 3G, H and 4F, G). Differently, MEL treatment after H$_2$O$_2$ exposure determined an apoptotic/necrotic cell increase, respect to H$_2$O$_2$ alone. In fact, percentages detected were 31% of PI/FITC ANX-V positive events (Figure 2E). A higher number of apoptotic cells could also be revealed at RM, LM and TEM, generally characterized by late apoptotic pattern and secondary necrosis (Figure 3I, L and 4H, I).

**Discussion**

MEL is an effective antioxidant molecule and a free radical scavenger. Its efficacy is also correlat-

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**Figure 1.** MTT assay test. A high percentage of cell vitality appears in both control and MEL treated cells (100% and 95.4% respectively). A reduction of cell vitality (75.7%) was obtained after H$_2$O$_2$ treatment. When cells were pre-treated with 1 mM MEL their vitality increases to 83.9%, whereas when MEL was added after H$_2$O$_2$ exposure a behaviour similar to H$_2$O$_2$ treatment (74%) was observed. Data were collected from three experimental conditions and they were expressed as mean +/- standard error.

**Figure 2.** FC analysis of DNA content. U937 control cells and cells treated with 1mM MEL alone show a similar distribution of cell cycle phases (A and B, respectively). H$_2$O$_2$ treated cells reveal a weakly sub-diploid peak (C), drastically reduced when cells were MEL-pretreated (D). On the contrary, an evident increase of subG1 events appears when U937 cells are incubated with 1 mM MEL after H$_2$O$_2$ exposure (E). Representative cyto-grams are taken from three independent experiments for all experimental conditions.
ed to ability to enhance the activities of a variety of antioxidative enzymes (Rodriguez et al., 2004), stimulatory actions on glutathione synthesis (Winiarska et al., 2006), reduction of electron leakage from the mitochondrial electron transport chain (Leon et al., 2006), and to its synergic interactions with other antioxidants (Lopez-Burillo et al., 2003).

MEL was originally shown to inactivate the highly toxic hydroxyl radical (•OH). Since this discovery, its scavenging repertoire has been demonstrated against H₂O₂ (Tan et al., 2000), hypochlorous acid (HOCl) (Zavodnik et al., 2004), singlet oxygen (O₂•) (Matuszak et al., 2003), superoxide anion radical (O₂•⁻), nitric oxide (NO•) (Aydogan et al., 2006), peroxynitrite anion (ONOO⁻) (Reiter et al., 2001) and others (Hardeland et al., 2005).

In addition, it also favours the defence against oxidative stress by promoting enzymes that metabolize radicals and their products to innocuous agents (Reiter et al., 2008). These indirect MEL antioxidative effects may be mediated via membrane and/or nuclear receptors. Examples

Figure 3. U937 cells at RM (A, C, E, G, I) and LM (B, D, F, H, L). Control cells (A, B) and MEL alone treated cells (C, D) show a well preserved morphology. After H₂O₂ treatment, apoptotic cell death is evident (E, F). When MEL is added before H₂O₂ treatment, only few cells appear apoptotic, being the others similar to the control (G, H). When MEL is added after H₂O₂ exposure several apoptotic cells are observed (I, L). Bar = 10 µm.
include the widely reported stimulation of glutathione peroxidase (GPx) (Rodriguez et al., 2004), which converts hydroperoxides, including H$_2$O$_2$, to water and oxygen, while oxidizing GSH.

In this study we have examined the effect of MEL on apoptosis induced via oxidative stress generated by H$_2$O$_2$. Our data show that H$_2$O$_2$, while being a free radical inducer, can be considered not only a cytotoxic agent, but an important apoptotic trigger, if utilized at proper concentration i.e. 500 µM. In addition, this study confirms MEL action as an effective antioxidant and free radical scavenger, with a possible protective role from apoptotic/oxidative damage.

These findings are in agreement with our previous study (Luchetti et al., 2006), which reported that MEL was able to prevent UVB-induced apoptosis in U937 cells. Both UVB radiations and H$_2$O$_2$ lead to ROS generation and cell damage, but UVB seem a more powerful apoptotic trigger. To this

![Figure 4. TEM analysis. Control cells (A) and U937 treated with MEL alone (B) show a comparable ultrastructural good morphology. After 500 µM H$_2$O$_2$ treatment, apoptosis appears in all apoptotic stages (C, D, E). Pre-treatment with MEL and successive exposure to H$_2$O$_2$ evidences a decrease of apoptotic cell number (F, G). When cells are exposed to H$_2$O$_2$ and then treated with MEL, apoptotic cell number appears strongly increased, with cells in late apoptotic stages, micronuclei, and secondary necrosis (H, I). A, C, F, H, bar = 2.5 µm. B, D, F, G, I bar = 2 µm.](image-url)
regard, a study on keratinocytes demonstrated that both UVB and H2O2 led to the increase of intracellular H2O2 levels, the antioxidants catalase and glutathione monoester, inhibited apoptosis only when induced by H2O2 and not by UVB (Chang et al., 2003). In addition, DNA damage in the form of cyclobutane pyrimidine dimers was observed after exposure to UVB, but no photo-products were found in H2O2-treated cells, suggesting a ROS-independent pathway of UVB-induced apoptosis (Chang et al., 2003). In the present study apoptosis is a weak process if compared with that induced by UVB.

Nevertheless, Luchetti et al., (2006) showed that apoptosis was reduced when MEL is added both before and after UVB exposure. Differently, in the present study MEL prevents apoptosis only when added before H2O2. Differently, MEL incubation after H2O2 treatment lead to an increase of apoptotic cell death, suggesting that H2O2 intracellular level increases and free radical production can not be further controlled by MEL. A recent work on mouse mitochondria incubated with MEL, evidenced its capacity to down regulate oxygen consumption, to inhibit oxygen flux increase in the presence of an excess of ADP, to reduce membrane potential, and, consequently, to prevent superoxide anion and hydrogen peroxide production (Lopez et al., 2009).

This study emphasizes MEL role as effective antioxidant and anti-apoptotic trigger. In fact, besides its direct scavenging actions, MEL stimulates several antioxidative enzymes, including superoxide dismutase, glutathione peroxidase and glutathione reductase (Reiter et al., 2002). Therefore, in our model MEL pre-treatment prevents H2O2 induced apoptosis because of MEL capacity to stimulate protective oxygen radical scavengers. Apoptosis induced by cell damage, via intrinsic pathway, implies redox imbalance or even direct oxidative stress (Gao et al., 2005). Therefore, radical-scavenging MEL property is the mechanism correlated to its anti-apoptotic activity, confirmed by the observation that MEL concentrations required for radical scavenging are closely compared to the anti-apoptotic ones (Radogna et al., 2007). In conclusion, this study is a demonstration of MEL protective role from ROS increase induced by H2O2, at apoptotic concentration.

References

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