Neuroprotection by Melatonin on Astrocytoma Cell Death

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Glial cells play an active role in the homeostatic regulation of the central nervous system (CNS). Astrocytes, the most abundant glial cell types in the brain, provide mechanical and metabolic support for neurons. The regulation of astrocyte apoptosis, therefore, is important for physiological and pathological processes in the CNS. Melatonin is a neurohormone that regulates target cells via binding to specific high-affinity plasma membrane receptors, MT1/MT2. In addition to regulating circadian rhythms, melatonin has recently attracted much interest for its potential regulation of cell apoptosis. We recently showed that melatonin antagonizes apoptosis on U937 cells via intersecting signal transduction events involving binding to MT1/MT2 and activation of lipoxygenase. Here we describe the neuroprotective potential of melatonin, showing that melatonin significantly reduces damage-induced apoptosis in astrocytoma cells. The mechanism of protection is different from that shown in U937 cells, because it does not involve MT1/MT2 or lipoxygenase; likewise, Ca^{2+} influx is not involved. Intriguingly, inhibition of phospholipase C (PLC) with neomycin reverses melatonin protection, suggesting that a PLC-dependent signal transduction, different from that triggered by MT1/MT2, is involved in the antiapoptotic pathway of melatonin.

Key words: **melatonin; apoptosis; astrocytoma**

Introduction

Glial cells, including astrocytes and microglial cells, are well-known protectors of neurons.[1,2] Astrocytes contribute to neuroprotection and preserve neuron survival; thus, any astrocytic dysfunction seriously affects neuronal viability.[3] Apoptosis is an essential physiological process for maintenance of brain homeostasis and plays a critical role in ischemia, neurodegenerative disease, and brain tumors.[3,4]

Melatonin plays a central role in circadian rhythms and regulation of seasonal reproductive cycles in vertebrate physiology.[5] It is biosynthesized from L-tryptophan by a multistep enzymatic reaction in pinealocytes[5] and leucocytes.[6] This neurohormone is involved in many regulatory functions of the cells, possibly through receptor engagement; that is, it modulates the immune response,[6] regulates the apoptotic response on several cell types,[7] and regulates signal transduction reactions.[8] Three molecular structure–based subtypes of the melatonin receptors have been described: Mel_{1A}, or MT1 (expressed in mammalian and bird brain); Mel_{1B}, or MT2 (expressed mainly in mammalian retina); and Mel_{1C}, or MT3 (found in amphibian melanophores, brain, and retina, as well as in bird and fish brain).[9] Melatonin receptors regulate several second messengers: cAMP, cGMP, diacylglycerol, inositol trisphosphate, arachidonic acid, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[8]

Melatonin is an endogenous free-radical scavenger and an antioxidant in many systems,

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especially effective in reducing/preventing oxidative damage in the brain.[10–12] Melatonin protects neurons from cell death induced by several neurotoxins at pharmacological and physiological doses.[13–15]

However, the mechanism by which melatonin prevents apoptosis on the central nervous system (CNS) is not clear. Thus, it is important to understand whether the neuroprotective effect of melatonin derives from its ability to engage specific receptors[5] or from its antioxidant ability to directly pass the blood–brain barrier and scavenge free radicals.[5,16,17]

In this study, we analyzed the protective effect of melatonin in cultured astrocytoma cells induced to apoptosis by the cell-damaging agent puromycin (PMC), focusing on the role of receptor interaction and the subsequent signal transduction pathway as possible mechanisms involved, in view of the important therapeutic potential that melatonin may exert in the treatment of neurodegenerative disease.

Materials and Methods

Cell Culture

Human astrocytoma cell line 132 1N1 was maintained in 25-cm^2 flasks in Dulbecco's modified Eagle medium with 10% inactivated fetal calf serum, 2 mmol/L L-glutamine, and 100 IU/mL penicillin–streptomycin, and cells were kept in a controlled-atmosphere (5% CO_2) incubator at 37°C. Cells were trypsinized weekly and reseeded for experiments. Medium was renewed every 2–3 days and after 7–8 days the cells became subconfluent and ready for use.

Treatments

Melatonin was used at the final concentration of 1 mmol/L, unless otherwise specified, and added to the culture medium 1 h before apoptosis induction. Melatonin action on MT1/MT2 receptors was antagonized by 2-benzyl-*N*-acetyltryptamine (luzindole). Luzindole was added at the concentration of 50 μmol/L, 30 minutes before other treatments. For phospholipase C (PLC) inhibition, neomycin was used at 100 μmol/L, 30 min before other treatments. We added 10 μmol/L nifedipine, a Ca^{2+} flux inhibitor, 30 min before other treatments. For lipoxygenase (LOX) inhibition, the specific 5-LOX inhibitor AA861 was used at 20 μmol/L and added 30 min before other treatments.

Induction of Apoptosis

Apoptosis was induced by the protein synthesis inhibitor PMC (10 μg/mL). Apoptosis was evaluated after 15 h of PMC incubation.

Identification and Quantification of Apoptotic Cells

Nuclear morphology of control and treated cells was analyzed by fluorescence microscopy after staining with Hoechst 33342; apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Apoptosis was evaluated among the Hoechst-stained cells by counting at least 300 cells in at least three randomly selected fields.

Results

Melatonin Reduces Apoptosis in Astrocytoma Cells

We investigated melatonin's effects on stress-induced apoptosis. Figure 1A shows that melatonin reduces PMC-induced apoptosis in a dose-dependent fashion, protection being highly significant for the 1 mmol/L dose. The doses required for melatonin's antiapoptotic effect ($\geq$10 μmol/L) are not compatible with MT1/MT2 receptor stimulation because the melatonin receptor affinity is 1 nmol/L. To definitely exclude that melatonin's protective effect may depend on receptor interaction, we used luzindole, a specific melatonin membrane receptor antagonist. Luzindole is an important tool in

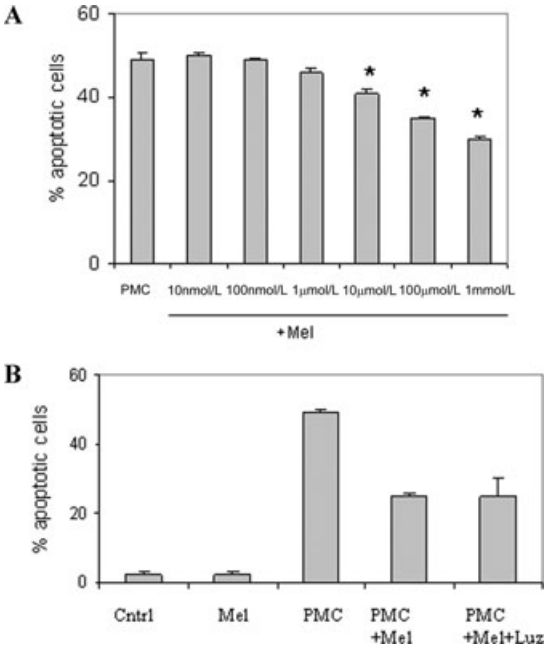

Figure 1. Melatonin protects astrocytoma cells from apoptosis in a receptor-independent way. (**A**) Astrocytoma cells were incubated in complete medium (see Materials and Methods) with different doses of melatonin (Mel) for 1 h, followed by PMC (10 μg/mL). Apoptosis was evaluated at 15 h. Results are the mean ± SD of at least five independent experiments. Apoptosis reduction is significant starting from melatonin 10 μmol/L (*, $P < 0.05$). (**B**) Melatonin's (Mel) antiapoptotic effect is sensitive to luzindole (Luz). Luzindole 50 μmol/L was added 30 min before incubation with melatonin 1 mmol/L and then apoptosis was induced with PMC (10 μg/mL). Apoptosis was estimated at 15 h. Results are the mean ± SD of at least three independent experiments. Cntrl, control.

melatonin research because it specifically inhibits G protein activation by a melatonin-engaged MT1 or MT2 receptor.[18] Figure 1B shows that luzindole does not inhibit melatonin's antiapoptotic effects. Thus, this result confirms that melatonin can protect astrocytoma cells from apoptosis in a receptor-independent way.

Melatonin's Protective Effect Is Sensitive to Neomycin

In U937 monocytes, melatonin exerts an antiapoptotic effect by the interaction of two independent pathways: one involving MT1/MT2 receptor interaction[21] and the other involving LOX (Radogna *et al.*, in preparation). To understand whether in astrocytoma cells LOX was also involved in melatonin protection, we investigated whether AA861, a specific 5-LOX inhibitor, could inhibit melatonin protection. Figure 2A shows that, unlike U937, AA861 had no effect on melatonin protection of astrocytoma cells.

Instead, we found that neomycin, which inhibits PLC by protecting its substrate PIP2, partially inhibits melatonin protection (Fig. 2B), suggesting a role of PLC in the antiapoptotic pathway of melatonin. Activation of PLC stimulates the production of diacylglycerol and IP3,[19] which in turn stimulates Ca^{2+} influx from the extracellular environment. Because melatonin mobilized Ca^{2+} in other cell systems,[20] and because this plays a role in melatonin protection from apoptosis in U937 cells,[21] we investigated the involvement of Ca^{2+} influx in melatonin protection on astrocytoma cells with the L-type Ca^{2+} channel inhibitor nifedipine. Figure 2C shows that nifedipine did not inhibit melatonin protection. Thus, Ca^{2+} influx is not involved in the antiapoptotic effects of melatonin.

Discussion

Glial cells play an active role in CNS development and are essential for neuronal differentiation and neuronal survival.[2] Thus, the study of the modulation of apoptosis in astrocytes is particularly important because any changes in astrocyte shape and function seriously affect neuronal viability.[3,4] Apoptosis has been associated with a variety of acute disease and chronic pathologies in which neuronal cell death correlates with an increase of oxidative stress.[22–24] With the goal of clarifying the therapeutic potential of melatonin in neurodegenerative disease and neurological disorder, we investigated the role of melatonin on apoptosis of astrocytoma cells.

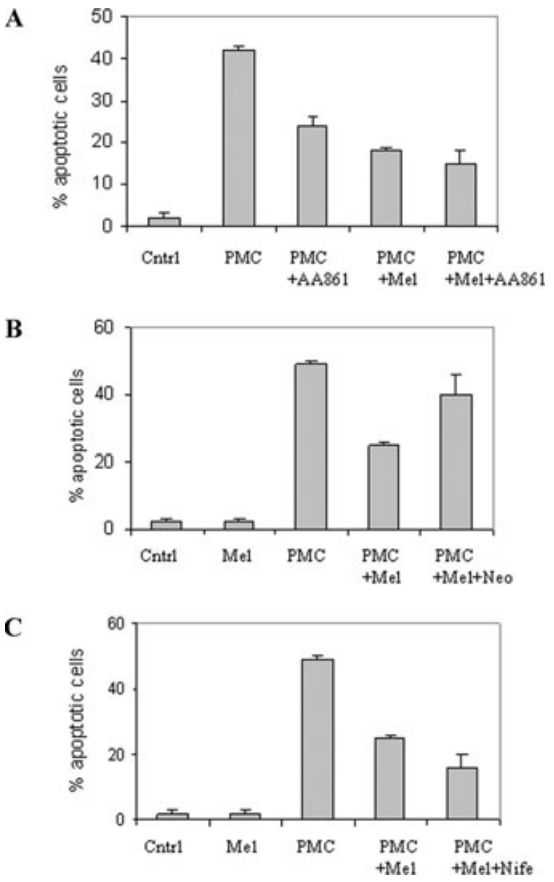

Figure 2. Melatonin's (Mel) protective effect requires PLC but not Ca^{2+} fluxes and LOX. (**A**) LOX is not required for melatonin's antiapoptotic effect. AA861 20 μmol/L, a 5-LOX inhibitor, was added 30 min before melatonin 1 mmol/L treatment, and then apoptosis was induced with PMC (10 μg/mL). Results are the mean ± SD of at least three independent experiments. (**B**) Astrocytoma cells were incubated with neomycin 100 μmol/L, a PLC inhibitor, added 30 min before melatonin 1 mmol/L treatment, and then apoptosis was induced with PMC (10 μg/mL). Results are the mean ± SD of at least three independent experiments. (**C**) Astrocytoma cells were incubated with nifedipine 10 μmol/L, an inhibitor of Ca^{2+} fluxes, added 30 min before melatonin 1 mmol/L treatment, and then apoptosis was induced with PMC (10 μg/mL). Results are the mean ± SD of at least three independent experiments. Cntrl, control.

We recently demonstrated that melatonin exerts a similar protective effect on U937 monocytes, at the same doses.[21] In that cell system, the signal transduction elicited by melatonin MT1/MT2 receptor stimulation is the mechanism through which melatonin exerts its antiapoptotic effects.[21] Here, we show that melatonin prevents apoptosis induced by the cell-damaging agent PMC also in astrocytoma cells. Thus, we investigated whether the antiapoptotic effect of melatonin in astrocytoma cells could follow the same mechanisms. Unexpectedly, we found that in astrocytoma cells the mechanism is different. The doses required for the antiapoptotic effect (≥10 μmol/L) are not compatible with receptor stimulation because the melatonin receptor affinity is 1 nmol/L. Moreover, the MT1/MT2 antagonist luzindole does not revert the effect. Thus, in astrocytoma cells melatonin's antiapoptotic effect occurs in a receptor-independent way. However, melatonin may mobilize other signaling pathways because the inhibition of PLC, a key actor in intracellular receptor–induced signaling, is sufficient to reverse melatonin's antiapoptotic effect in astrocytoma cells. However, this PLC protective pathway does not involve Ca^{2+} flux, as was instead demonstrated in U937 cells.[21]

In U937 monocytes, we found that melatonin's antiapoptotic effect requires the cooperation of additional mechanisms involving LOX (Radogna *et al.*, in preparation). However, unlike U937, the 5-LOX inhibitor AA861 does not reverse melatonin's antiapoptotic effect, showing that 5-LOX is not involved in astrocytoma protection.

These findings demonstrate a clear protective effect of melatonin in astrocytoma cell death that is different from what we reported for leukocytes. Thus, one must consider that melatonin may exert different effects depending on the cell type under study and that even if the result is similar, it may occur according to different mechanisms. In astrocytoma, antagonism of apoptosis occurs in a LOX-independent, MT1/MT2 receptor–independent way, but with possible involvement of the PLC pathway. We cannot rule out other possible explanations; indeed, neomycin may have other cellular targets, and other means of PLC inhibition will help to identify more clearly the role of PLC.

Moreover, we must consider the possible role of melatonin as an antioxidant in counteracting apoptosis. Some groups indeed reported protection against programmed cell death in neuronal cells by melatonin's antioxidant activity.[13–15] Further investigations are under study to elucidate the mechanism of this effect, because identifying the mechanism of melatonin's protective effect may help generate novel strategies for treating CNS injuries.

Conflicts of Interest

The authors declare no conflicts of interest.

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