Melatonin Attenuates Autophagy-like Cell Death Through Antioxidant Enzymes in Cerebellar Purkinje cells Following Asphyxial Cardiac Arrest

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Introduction

Transient global cerebral ischemia resulting from asphyxial cardiac arrest and cardiopulmonary resuscitation (CA/CPR) is an established model for the investigation of brain injury. Rodent models of global cerebral ischemia have been extensively used to examine injury mechanisms and neuroprotective therapies in delayed neuronal death of selectively vulnerable areas of the brain, such as the cerebral cortex, striatum and hippocampus. However, few studies have focused on the cerebellum following global ischemia, despite data indicating that the cerebellum is vulnerable to cerebral ischemic damage *in vivo* and *in vitro*.

Melatonin (N-acetyl 5-methoxytryptamine) is primarily synthesized in the pineal gland and is recognized as an interesting compound with physiological activity. Along with the relevant physiological roles, it has been reported that melatonin is capable of protecting the brain against ischemia/reperfusion injury by reducing oxidative damage.

The effects of melatonin are mediated by two main pathways that include receptor-mediated and non-receptor mediated effects. The two major membrane-associated melatonin receptors, MT1 and MT2, belong to the G-protein-coupled receptor superfamily, and are ubiquitously distributed in the central nervous system (CNS) as well as the peripheral organs.

Although little is known about the influence of melatonin receptors on neuronal signaling during ischemic conditions, it is suggested that the melatonin receptor pathway may play roles in the neuroprotective effects of melatonin against asphyxial CA/CPR-induced injury.

Experimental Purpose

To date, however, the contribution of autophagy to cerebellar Purkinje cell death following asphyxial CA/CPR remains unclear. Therefore, in the present study, we attempted to identify the time course of cerebellar Purkinje cell death following asphyxia CA/CPR using cresyl violet staining, calbindin-D 28k immunohistochemistry and Fluoro-Jade B (F-J B) histofluorescence. In addition, we focused on the role of melatonin in cerebellar Purkinje cell death, autophagy modulation and its relationship with antioxidant enzymes following asphyxia CA/CPR, utilizing western blot, immunohistochemistry and transmission electron microscopy in rat.

Material and Methods

Experimental animals

- Male Sprague-Dawley rats (10 weeks old, 300-310 g weight)
- 1) Vehicle-treated sham-operated group
- 2) Vehicle-treated CA-operated group
- 3) Melatonin-treated sham-operated-group
- 4) Melatonin-treated CA-operated-group
- 5) 4P-PDOT plus melatonin-treated sham-operated-group
- 6) 4P-PDOT plus melatonin-treated CA-operated-group
- 4P-PDOT, MT2 melatonin receptor antagonist; n = 7 in each group

Tissue processing for histology

Histostaining & histofluorescence

CV (cresyl violet); Fluoro-Jade B (F-J B); DHE Mouse anti-NeuN, calbindin-D 28k; Sheep anti-SOD1, GPx

Immunohistochemistry & Western blot

Mouse anti-NeuN, calbindin-D 28k; Sheep anti-SOD1, GPx

Experimental processing

Results

Fig. 1. Representative images are shown the CV staining in the cerebellum following asphyxial CA/CPR at sham (a, e), 12 hrs (b, f), 1 day (c, g) and 2 days (d, h). The numbers of CV + cells are significantly decreased in the Purkinje cell layer (PCL, arrows) at 2 days after asphyxial CA/CPR. GCL, granular cell layer; MCL, molecular cell layer. Scale bar = 800 (a - d) and 50 (e - h) μ m.

Fig. 2. Representative images are shown calbindin-D 28k immunohistochemistry (A) and F-J B histofluorescence staining (B) in the cerebellar vermis of the CA- and melatonin-treated CA-group at sham (a, e), 12 hrs (b, f), 1 day (c, g) and 2 days (d, h) after asphyxial CA/CPR. In the CA-group, calbindin D 28k+ cells (black arrows) are decreased in the Purkinje cell layer (PCL) 2 days after asphyxial CA/CPR, and many F-J B+ (white arrows) cells are increased in the PCL from 12 hrs; however, in the melatonin-treated CA-group, NeuN and F-J B staining is similar to that in the sham group. GCL, granular cell layer; MCL, molecular cell layer. Scale bar = 50 μ m; The quantitative graph is shown calbindin-D 28k+ (C) and F-J B+ (D) cells in the PCL. The bars are reported as means $\pm$ SEM from 3 independent experiments (n = 7, *p < 0.05 vs. sham-group; #p < 0.05 vs CA-group).

Fig. 3. (A) Representative western blots are shown relative melatonin receptors levels from the cerebellar vermis of the CA- and melatonin-treated CA-group at sham after asphyxial CA/CPR. (B) Relative band intensity of MT1 and MT2 level is measured by densitometer. Expression of MT2 protein is higher than expression of MT1 protein. Protein expression is normalized to β -actin. The bars are reported as means $\pm$ SEM from 3 independent experiments (n = 7, *p < 0.05 vs. MT1). (C) Representative images are shown immunohistochemical staining for MT1 and MT2 in the cerebellar vermis of the CA- and melatonin-treated CA-group at sham after asphyxial CA/CPR. Strong MT2 immunoreactivity is detected in the Purkinje cell layer (PCL). GCL, granular cell layer; MCL, molecular cell layer. Scale bar = 50 μ m.

Fig. 4. (A) Representative images are shown immunohistochemical staining for SOD1 in the cerebellar vermis of the CA- and melatonin-treated CA-, and 4P-PDOT+melatonin-treated CA-groups at sham (a, e, i), 12 hrs (b, f, j), 1 day (c, g, k) and 2 days (d, h, l) after asphyxial CA/CPR. In the CA- and 4P-PDOT+melatonin-treated CA-group, SOD1 immunoreactivity is apparently decreased in the Purkinje cells (black arrows) from 12 hrs post-asphyxial CA/CPR, and thereafter, the immunoreactivity in the Purkinje cells was hardly detected at 2 days after asphyxial CA/CPR; however, the distribution pattern of SOD1+ cells in the cerebellar Purkinje cells of the melatonin-treated CA-group is similar to that in the sham-group. GCL, granular cell layer; MCL, molecular cell layer; PCL, Purkinje cell layer. Scale bar = 50 μ m.

Fig. 5. Effect of melatonin on glutathione peroxidase (GPx) in the cerebellar vermis after asphyxial CA/CPR.

(A) Representative images are shown immunohistochemical staining for GPx in the cerebellar vermis of the CA- and melatonin-treated CA-, and 4P-PDOT+melatonin-treated CA-groups at sham (a, e, i), 12 hrs (b, f, j), 1 day (c, g, k) and 2 days (d, h, l) after asphyxial CA/CPR. In the all groups, GPx immunoreactivity is apparently detected around the Purkinje cell layer (PCL, black arrows) of the cerebellar vermis, and the pattern of GPx immunoreactivity is not significantly changed after asphyxial CA/CPR. GCL, granular cell layer; MCL, molecular cell layer. Scale bar = 50 μ m.

Conclusion

This is the first study evaluating cerebellar Purkinje cell death via the activation of autophagy using a rat model of asphyxial CA/CPR. Furthermore, our present study also shows that melatonin prevented this cerebellar Purkinje cell death through a receptor-dependent mechanism that upregulated synthesis of the antioxidant enzymes. Therefore, these results suggest a novel mechanism by cerebellar Purkinje cell death, as well as overexpression of antioxidant enzymes by melatonin treatment, might reduce ischemia/reperfusion injury via the inhibition of autophagy activation following asphyxial CA/CPR. Considering melatonin's high efficacy, these beneficial properties makes melatonin suitable as a potential treatment against brain ischemia injury.