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Melatonin inhibits the caspase-1/cytochrome *c*/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis

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ABSTRACT

Caspase-mediated cell death contributes to the pathogenesis of motor neuron degeneration in the mutant SOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis (ALS), along with other factors such as inflammation and oxidative damage. By screening a drug library, we found that melatonin, a pineal hormone, inhibited cytochrome *c* release in purified mitochondria and prevented cell death in cultured neurons. In this study, we evaluated whether melatonin would slow disease progression in SOD1^{G93A} mice. We demonstrate that melatonin significantly delayed disease onset, neurological deterioration and mortality in ALS mice. ALS-associated ventral horn atrophy and motor neuron death were also inhibited by melatonin treatment. Melatonin inhibited Rip2/caspase-1 pathway activation, blocked the release of mitochondrial cytochrome *c*, and reduced the overexpression and activation of caspase-3. Moreover, for the first time, we determined that disease progression was associated with the loss of both melatonin and the melatonin receptor 1A (MT1) in the spinal cord of ALS mice. These results demonstrate that melatonin is neuroprotective in transgenic ALS mice, and this protective effect is mediated through its effects on the caspase-mediated cell death pathway. Furthermore, our data suggest that melatonin and MT1 receptor loss may play a role in the pathological phenotype observed in ALS. The above observations indicate that melatonin and modulation of Rip2/caspase-1/cytochrome *c* or MT1 pathways may be promising therapeutic approaches for ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a progressive and selective loss of motor neurons, resulting in progressive paralysis and death (Rowland and Shneider, 2001). Although the exact pathophysiological mechanisms are not fully understood, the activation of caspase-mediated cell death pathways plays an important role in neuronal death in ALS patients and animal models (Friedlander, 2003; Li et al., 2000). The potential importance of apoptotic pathways in ALS is suggested by several observations: altered Bcl-2-family proteins result in a predisposition towards cell death; increased expression or activation of caspase-1 and caspase-3 in ALS; and appearance of morphological features in dying motor neurons that are reminiscent of apoptosis (Pasinelli et al., 2000; Sathasivam et al., 2001; Vukosavic et al., 1999). Further supporting evidence is the occurrence of apoptosis-related mitochondrial dysfunction in the spinal cord associated with disease progression (Dupuis et al., 2004; Shi et al., 2010). Consistent with the

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morphological/functional abnormalities of mitochondria in ALS, cytochrome *c* and other pro-apoptotic proteins are released into the cytoplasm. Moreover, in ALS animal models, disease onset can be delayed and lifespan can be prolonged by transgenic inhibition of caspase-1, by intracerebroventricular administration of specific and broad caspase inhibitors, by overexpression of Bcl-2, or by administering drugs that inhibit the release of cytochrome *c* (Friedlander et al., 1997a; Kostic et al., 1997; Li et al., 2000; Zhang et al., 2003a; Zhu et al., 2002).

Melatonin, a naturally occurring pineal hormone, is best known for its role in regulating the light-dark cycle. Many biological effects of melatonin depend upon its binding to specific receptors, although the antioxidant activity is also important to its physiological functions (Hardeland, 2005). To date, three types of melatonin receptors have been cloned, although only two, melatonin receptor 1A (MT1) and melatonin receptor 1B (MT2), are detected in mammalian neurons (Mazzucchelli et al., 1996; Naji et al., 2004). Abnormal expression of the MT1/MT2 receptor has been reported in several neurological diseases (Sanchez-Hidalgo et al., 2009; Savaskan et al., 2001; Wang et al., 2011), however, there are no reports evaluating the importance or the role of melatonin receptors in ALS.

We recently identified melatonin as an anti-apoptotic agent. As previously described (Wang et al., 2008), a library of 1040 FDA-approved drugs and bioactive chemicals was screened for their ability to inhibit calcium-mediated cytochrome *c* release from purified mitochondria. Twenty-one compounds were further tested in a neuronal cell death model. Melatonin was one of the most effective candidates at inhibiting cytochrome *c* release and preventing neuronal death. In the present study, we evaluated the protective effects of melatonin in a transgenic mouse model of ALS, which expresses mutant-human copper-zinc superoxide dismutase ($mSOD1^{G93A}$). Melatonin was indeed beneficial in this animal model, and its neuroprotection was associated with its inhibition of the caspase-1/cytochrome *c*/caspase-3 pathways. Furthermore, for the first time, we detected a significant increase of receptor interacting protein-2 (Rip2), reduction of melatonin levels, and a down-regulation of MT1 in the spinal cord of ALS mice. These disease-associated changes were ameliorated in ALS mice by exogenous melatonin supplementation.

Materials and methods

Animals and drug treatment

Transgenic ALS littermate mice expressing mutant human SOD1 G93A in the B6SJL genetic background were randomly assigned among experimental groups (Jackson Laboratory, Bar Harbor, ME), while controlling each group so that the gender ratio was always the same (Ryu et al., 2005; Veldink et al., 2003). Mice were maintained in a pathogen-free environment and provided food and water *ad libitum*. All of the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Melatonin was dissolved in 0.9% saline after being dispersed by dimethyl sulfoxide (DMSO) and prepared fresh daily. Melatonin (30 mg/kg, 10 μl/g body weight) was injected intraperitoneally (i.p.) daily beginning at six weeks of age until one day before sacrifice. Vehicle-control ALS mice were injected in the same manner with the DMSO/saline vehicle.

Clinical assessment

Beginning at 6 weeks of age, mice were weighed and assessed using neurobehavioral tests (n = 15 animals in each group). Muscle strength and coordination deficits were evaluated weekly using a Rotarod apparatus (Columbus Instruments, Columbus, OH) as previously described (Li et al., 2000; Wang et al., 2007; Zhu et al., 2002). Testing was terminated either when the mice fell from the rod or at 7 min if the mouse remained on the rod. Disease onset was defined as the first day when the mouse could not remain on the Rotarod for 7 min at the speed of 15 rpm. Mortality was scored at the age of natural death or the age when the mouse was unable to right itself within 30 s (surrogate death). Three times per week, the mice were evaluated for signs of motor deficits according to a four-point neurological scoring system (Shefner et al., 2001; Veldink et al., 2003; Weydt et al., 2003): no sign of motor dysfunction (0); evident tremor and inability to extend hind limbs when suspended by the tail (1); paresis of one or both hind limbs and presence of gait abnormalities (2); paralysis of one or both hind limbs and gait with dragging of at least one hind limb (3); inability to right itself within 30 s (4). Body weight was monitored weekly. All studies were performed by investigators blinded to treatment.

Histopathological evaluation and immunohistochemistry

At 120 days of age, a separate cohort of mice (n = 5 animals in each group) was anesthetized, transcardially perfused with 4% paraformaldehyde, and spinal cords were harvested. After post-fixation and cryoprotection, serial sections of the frozen tissue were cut in the coronal plane at 50 μm intervals as described previously (Wang et al., 2007). Motor neuron survival was evaluated by Nissl staining. The absolute numbers of motor neurons with visible nucleoli were counted in the ventral horns of all sections from lumbar regions at the L2–3 level. The observer was blinded to the identity of each preparation. The numbers reported for each experimental group were average counts of motor neurons per section (including right and left ventral horns) among 20 continuous sections from each of five animals. The area of grey and white matter and the total cross-sectional area were also measured in the same Nissl-stained preparations of lumbar spinal cord using Spot RT Software (Diagnostic Instrument, Inc., Sterling Heights, MI). Subsequently, the volume per section was calculated as area × thickness. An average volume per section was calculated for mice in each experimental group.

In parallel experiments, serial sections of spinal cord were immunostained with antibodies specifically against cytochrome *c* (Zymed Laboratories, South San Francisco, CA), activated caspase-3 (BD Pharmingen, San Jose, CA), Ricinus communis agglutinin-1 (RCA-1, Sigma), glial fibrillary acidic protein (GFAP, BD Pharmingen, San Jose, CA), or MT1 (Santa Cruz Biotechnology, Inc, CA) respectively followed by conjugated secondary antibody as previously described (Ferrante et al., 1997; Ryu et al., 2005).

Tissue fractionation and western blotting

Mice were sacrificed at the age of 120 days, and samples of total lysate and cytosolic fraction were prepared from spinal cords as described previously (Li et al., 2000; Zhang et al., 2011; Zhu et al., 2002). Briefly, for the samples of total protein lysates, tissue samples (mouse or human spinal cord specimens) were homogenized on ice in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 142.5 mM KCl, 5 mM MgCl2, 10 mM Hepes, pH 7.4) with protease inhibitor cocktail (Sigma, St. Louis, MO) and PMSF (0.1 mg/ml; Fluka, Switzerland). Lysates were centrifuged twice at 10,000 ×*g* for 20 min at 4 °C. The resulting supernatants were analyzed by western blotting with antibodies to caspase-1, caspase-3, caspase-9, Rip2, MT1 and MT2. To prepare cytosolic fractions, tissue samples were gently homogenized in cold buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 2 mM EDTA, 1 mM DTT, 10 mM Hepes, pH 7.4, plus protease inhibitor cocktail and 0.1 mg/ml PMSF) and clarified by a 700 ×*g* centrifugation for 5 min at 4 °C. The supernatant was centrifuged