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Melatonin reduced microglial activation and alleviated neuroinflammation induced neuron degeneration in experimental traumatic brain injury: Possible involvement of mTOR pathway

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ABSTRACT

This study was designed to detect the modulation manner of melatonin on microglial activation and explore herein possible involvement of mammalian target of rapamycin (mTOR) pathway following traumatic brain injury (TBI). ICR mice were divided into four groups: sham group, TBI group, TBI + sal group and TBI + Melatonin group. A weight-drop model was employed to cause TBI. Neurological severity score (NSS) tests were performed to measure behavioral outcomes. Nissl staining was conducted to observe the neuronal degeneration and wet-to-dry weight ratio indicated brain water content. Immunofluorescence was designed to investigate microglial activation. Enzyme-linked immunosorbent assay (ELISA) was employed to evaluate proinflammatory cytokine levels (interleukin-beta (IL-1β), tumor necrosis factor-alpha (TNF-α)). Western blotting was engaged to analyze the protein content of mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase (p70S6K) and S6 ribosomal protein (S6RP). Melatonin administration was associated with markedly restrained microglial activation, decreased release of proinflammatory cytokines and increased the number of surviving neurons at the site of peri-contusion. Meanwhile, melatonin administration resulted in dephosphorylated mTOR pathway. In conclusion, this study presents a new insight into the mechanisms responsible for the anti-neuroinflammation of melatonin, with possible involvement of mTOR pathway.

1. Introduction

Traumatic brain injury (TBI) is the leading cause of death under the age of 40 in developed countries, and approximately 10 million persons are in hospital for TBI annually worldwide (Langlois et al., 2006). TBI cause primary mechanical injury of cerebral cells and also initiates secondary damage occur immediately following the primary damage. The secondary nonmechanical injury is progressive and lasts from hours to days (Cernak, 2005).There are several pathological process reported to be responsible for the neuronal death in the secondary damage of TBI (Yatsiv et al., 2005; Xu et al., 2013; Cornelius et al., 2013), including inflammation (Ziebell and Morganti-Kossmann, 2010; Xu et al., 2013). The activation of microglia is wildly accepted as a prominent histological evidence of brain injury (Morganti-Kossmann et al., 2007; Venkatesan et al., 2010). It is also thought as the major source of proinflammatory cytokines (Cao et al., 2012; Kelley et al., 2007; Morganti-Kossmann et al., 2007; Nakajima and Kohsaka, 2004; Zhang et al., 2013) after TBI. Now more and more researchers targeted it to retard the pathological process related inflammation after TBI (Sanchez et al., 2001; Ng et al., 2012; Siopi et al., 2012; D'Avila et al., 2012).

Melatonin (N-acetyl 5-methoxytryptamine) has been proved to be a neuroprotective agent in TBI (Tsai et al., 2011; Dehghan et al., 2013; Cirak et al., 1999; Beni et al., 2004). The anti-inflammation characteristic of melatonin have been accepted as a major protective mechanism for melatonin to brain injury (Tsai et al., 2011; Beni et al., 2004; Samantaray et al., 2009). However, the modulation manners of melatonin to microglial activation have not been particular investigated in the research with respect to TBI.

Mammalian target of rapamycin (mTOR) is a rapamycin sensitive serine/threonine protein kinase which plays a major role in modulating protein synthesis initiation (Dazert and Hall, 2011).

Abbreviations: TBI, traumatic brain injury; mTOR, mammalian target of rapamycin; NSS, neurological severity score; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-beta; TNF-α, tumor necrosis factor-alpha; p70S6K, p70 ribosomal S6 kinase; S6RP, S6 ribosomal protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick 3′-end labeling.

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Growth factors, hormones and nutrients activate the phosphoinositide 3-kinase pathway, leading to the phosphorylation and activation of mTOR on Ser2448 (Reynolds et al., 2002; Chong et al., 2010). Activated mTOR exerts its functions of stimulation of translation through phosphorylation and activation of p70 ribosomal S6 kinase (p70S6K), which in turn phosphorylates S6 ribosomal protein (S6RP) (Park et al., 2012). S6RP phosphorylation is a critical effector of mTOR in the regulation of cell proliferation and protein synthesis. The up-regulated expression of phospho-S6RP has been found in microglia rather than neuron after TBI (Park et al., 2012) and mTOR play a crucial role in microglial viability (Dello et al., 2009). Phosphorylation of mTOR pathway is a considerable reason for activation of microglia after TBI. Therefore, mTOR is a reasonable therapeutic target for inflammatory response after TBI. Thus, in the present study we explored the possible involvement of melatonin in modulation the activation of mTOR pathway in a mice TBI model and revealed a marked beneficial effect of melatonin on neurologic outcome associated its suppression microglia activation following TBI.

2. Materials and methods

2.1. Animals and experimental protocols

Male ICR mice (Experiment Animal Centre of Nanjing Medical University, Jiangsu, China) ages 6–8 weeks, weighing 28–32 g were used in this study. Experiment protocols were approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH). The mice were housed on a 12 h light/dark cycle circumstance with free access to food and water.

Male ICR mice were divided into four groups: sham group, TBI group, TBI + sal group and TBI + Melatonin group. In the animals of TBI + melatonin group, melatonin (10 mg/kg diluted in 3 mg/mL vehicle) was administered i.p. at 0, 1, 2, 3 and 4 h after TBI. Mice of TBI + sal group received equal volumes of vehicle (0.9% saline) at corresponding time points. Mortality rate: Sham group 0% (0 of 35 animals), TBI group 24% (11 of 46 animals), TBI + sal group 25% (15 of 60 animals) and TBI + Melatonin 22% (13 of 58 animals).

2.2. Model of TBI

The model of TBI employed in the present study was a weight-drop model previously described by Flierl et al. (2009). Mice were anesthetized with an intraperitoneal (i.p.) injection of chloral hydrate (1%, 5 mL/kg) and then placed onto the platform directly under the weight of the weight-drop device. A 1.5 cm midline longitudinal scalp incision was made and the skull exposed. After locating the left anterior frontal area (1.5 mm lateral to the midline on the mid-coronal plane) as the impact area, a 200 g weight was released and dropped onto the skull from a height of 2.5 cm. Mortality rate from apnea was reduced by early respiratory support. The scalp wound was closed with standard suture material, and the mice were returned to cages, where they had free access to water and food. Considered the circadian change of melatonin in organism, we chose to perform the TBI in same time period, during 14:00–16:00, which had showed a relatively constant level of melatonin in serum (Welp et al., 2010). Sham-injured animals underwent the same procedures but did not undergo the weight drop.

2.3. Brain tissue processing

For isolation of protein, animals were deeply anesthetized with a solution of chloral hydrate at 24 h after TBI and perfused intracardially with 30–40 mL of cold (4 °C) heparinized 0.9% saline. The left (ipsilateral) cerebral cortex peri-contusion was collected, immediately frozen in liquid nitrogen, and then transferred to a −80 °C freezer until use. For immunohistochemistry, animals were sacrificed 24 h after TBI. After being deeply anesthetized with chloral hydrate, animals were perfused intracardially with 30–40 mL of cold heparinized 0.9% saline followed by 20–30 mL of cold 4% paraformaldehyde. The whole brain was removed and immersed in 4% paraformaldehyde overnight. For immunofluorescence, the brain was subsequently immersed in 20% sucrose followed by 30% sucrose.

2.4. Western blot analysis

Protein concentrations were determined by the Bradford method. Equal amounts of protein (50 μg) per lane were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene-difluoride (PVDF) membranes. The membranes were blocked for 2 h in blocking buffer (Tris buffered saline/0.05% Tween 20 (TBST) containing 5% skim milk) and then incubated overnight at 4 °C with primary antibodies against mTOR (1:1000; Cell Signaling Technology, Danvers, MA, USA) , Phospho-mTOR (Ser2448) (1:1000; Cell Signaling Technology, Danvers, MA, USA) , p70 S6 Kinase (1:500; Anbo Biotechnology, San Francisco, CA, USA) , Phospho-p70 S6 Kinase (Ser371) (1:500; Anbo Biotechnology, San Francisco, CA, USA) , S6 Ribosomal Protein (1:1000; Cell Signaling Technology, Danvers, MA, USA) , Phospho-S6 Ribosomal Protein (Ser235/236) (1:2000; Cell Signaling Technology, Danvers, MA, USA) and GAPDH (1:5000; Bioworld Technology, Minneapolis, MN, USA) in blocking buffer. After being washed with TBST (3 × 10 min), the membranes were incubated with goat anti-rabbit horseradish peroxidase conjugated IgG (1:5000; Bioworld Technology) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Millipore, Billerica, MA, USA) and exposure to X-ray film. Developed films were digitized on an Epson Perfection 2480 scanner (Seiko Corp., Nagano, Japan). Band density was quantified by using Un-Scan-It 6.1 software (Silk Scientific Inc., Orem, UT, USA); data were normalized to GAPDH.

2.5. Immunofluorescence staining

For immunofluorescence, serial 8-μm coronal sections were obtained using a cryostat. Four sets of 5 evenly spaced (300 μm apart) sections spanning the injured cortex were collected from each brain. Based on the established immunostaining protocol, slides were incubated in blocking buffer (10% normal goat serum in PBS containing 0.1% Triton X-100) for 2 h followed by overnight incubation at 4 °C in primary antibodies (rabbit anti-IBA-1 (1:5000; Wako, Osaka, Japan) (alone staining) or goat anti-IBA-1 (1:200; Abcam, Cambridge, MA, USA) and rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (1:200; Cell Signaling Technology, Danvers, MA, USA) (double staining)). The next day, the slides were washed with PBS three times for 5 min and then incubated with appropriate secondary antibodies for 1 h at room temperature. The slides were washed three times in PBS, counterstained with DAPI for 2 min, and rinsed with PBS. Cover slips were applied with mounting media. Fluorescence was imaged on an Olympus IX71 inverted microscope system and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, USA). The specificity of the immunofluorescence reaction of the second antibody was evaluated by replacement of the primary antibody with PBS.

Microglial activation was scored on the basis of IBA-1 expression intensity, density of IBA-1 expressing cells, and microglial morphology, as detailed in Table 1. The scoring method comes from the article that previously published (Kauppinen et al., 2008).