Melatonin Attenuates the Cerebral Ischemic Injury via the MEK/ERK/p90RSK/Bad Signaling Cascade

Phil-Ok KOH1*

1)Department of Anatomy, College of Veterinary Medicine, Research Institute of Life Science, Gyeongsang National University, 900 Gajwa-dong, Jinju 660–701, South Korea

(Received 12 June 2008/Accepted 7 July 2008)

ABSTRACT. Melatonin prevents neuronal cell death in ischemic brain injury. This study investigated whether melatonin inhibits the apoptotic signal through the activation of Raf-MEK-ERK and its downstream targets, including 90 ribosomal S6 kinase (p90RSK) and Bad. Adult male rats were treated with melatonin (5 mg/kg) or vehicle prior to middle cerebral artery occlusion (MCAO). Brains were collected 24 hr after MCAO. We confirmed that melatonin significantly decreases the number of TUNEL positive cells in the cerebral cortex. Western blot analysis showed that levels of Raf-1, MEK1/2, and ERK1/2 phosphorylation decrease in vehicle-treated animals. Melatonin prevents the injury-induced decrease of Raf-1, MEK1/2, and ERK1/2 phosphorylation. Also, it inhibits the injury-induced decrease of p90RSK and Bad phosphorylation. Recently, we reported that melatonin prevents the injury-induced reduction of interaction between pBad and 14–3–3 and inhibits the activation of caspase-3. Subsequently, melatonin prevents the injury-induced increase of cleaved PARP levels. Taken together, these results suggest that melatonin prevents cell death resulting from ischemic brain injury, and that its neuroprotective effects are mediated by the activation of Raf/MEK/ERK/p90RSK cascade.

KEY WORDS: ERK, MEK, Melatonin, Neuroprotection, p90RSK.

Melatonin, produced primarily from the pineal gland, has a variety of physiological functions, including regulation of circadian rhythms related to sleep and season, removal of free radicals, and preventing the oxidation of biomolecules [11, 12, 21]. Moreover, melatonin exerts a neuroprotective effect against ischemic brain injury [3, 6, 7, 16], which has been linked to the suppression of apoptotic cell death and the activation of cell survival signals [8, 9, 17].

The mitogen-activated protein (MAP) kinase/extracellular-regulated kinase (ERK)1/2 signaling pathway mediates a number of cellular processes that include cell differentiation, growth, survival, and apoptosis [15]. Several growth factors stimulate a protein kinase cascade that sequentially activates Raf, MEK, and ERK1/2 [15]. The activation of Raf and MEK results in the phosphorylation of ERK1/2 [2, 4, 20]. Subsequently, ERK1/2 phosphorylates the 90 kDa ribosomal S6 kinase (p90RSK) and the transcription factor Elk-1. Phosphorylated p90RSK leads to the phosphorylation of Bad [2, 4, 20]. Phosphorylated Bad is sequestered in the cytosol by the binding of 14–3–3, 14–3–3 plays an anti-apoptotic role through interactions with pro-apoptotic molecules such as Bad and forkhead transcription factors [13]. However, dephosphorylated Bad promotes apoptotic cell death by interacting with Bcl-x(L) and releasing pro-apoptotic Bax from the binding of Bax and Bcl-x(L) [22, 24]. The released Bax promotes the release of cytochrome C from mitochondria and the activation of the caspase cascade, initiating nuclear DNA condensation and fragmentation [22, 24]. Activation of poly(ADP-ribose) polymerase (PARP), a nuclear protein, allows it to participate in DNA base excision repair in response to environmental stress [19]. PARP is important for cell viability, cleavage of PARP by cleaved caspases facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [14].

Previous work has demonstrated that melatonin plays a neuroprotective role in ischemic brain injury through ERK1/2 phosphorylation [7]. Although several studies have demonstrated the neuroprotective effects of melatonin, little data are available regarding the activation of ERK1/2 and its up- and down-stream targets. This study investigated the neuroprotective mechanism of melatonin through the activation of Raf-MEK-ERK and its downstream targets, p90RSK and Bad.

MATERIALS AND METHODS

Experimental animals and melatonin treatment: All animal procedures were carried out according to the Guide for the care and use of laboratory animals, published by the U.S. National Institutes of Health. Adult male Sprague–Dawley rats (250–260 g, 10 weeks age, n=40) were purchased from Samtako Co. (Animal Breeding Center, Korea) and were housed under controlled temperature (25°C) and lighting (12/12 light/dark cycle). Animals were randomly divided into two groups, vehicle-treated group and melatonin-treated group (n=20 per group). All experiments were performed during the day between 10:00 and 16:00 hr. Melatonin (Sigma, St. Louis, MO, U.S.A.) was dissolved in normal saline (1 ml) containing less than 5% dimethyl sulfoxide (Sigma) as the vehicle. A single dose of melatonin at 5 mg/kg or the vehicle alone was given via i.p. injection at 30 min before the onset of middle cerebral artery occlusion.
Middle cerebral artery occlusion: Before the operation to induce MCAO, animals were anesthetized with sodium pentobarbital (100 mg/kg). MCAO was induced as previously described [10]. The right common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A piece of 4/0 monofilament nylon suture with its tip slightly rounded by heat, was inserted through the right internal carotid artery to the base of the middle cerebral artery, thus occluding blood flow to the cortex and striatum. At 24 hr after the onset of permanent occlusion, animals were decapitated and their brains were rapidly removed.

TUNEL histochemistry: TUNEL histochemistry was performed using the DNA Fragmentation Detection Kit (Onogene Research Products, Cambridge, MA, U.S.A.). Brains were fixed in 4% neutral buffered paraformaldehyde solution, embedded with paraffin, and sectioned in 4 µm thick for TUNEL staining. Sections were incubated in equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme for 60 min at room temperature (R.T.). The reaction was terminated by incubation in stop buffer for 5 min at R.T. Sections were labeled with digoxigenin peroxidase and visualized with diaminobenzidine (DAB) (Sigma) substrate. Sections were counterstained with cresyl violet and were dehydrated in graded alcohol. Slides were observed under a microscope and then photographed. In the TUNEL stained sections, 5 fields for each section was selected from cerebral cortex. TUNEL-positive cells were quantified using light microscopy. The total cell number and TUNEL-positive cell number were obtained in each field. The percentage of TUNEL-positive cells is described as the percentage of the number of TUNEL-positive cells to the total number of cells in each field.

Western blot analysis: Brains were dissected into the ipsilateral and contralateral cortex. Tissues were snap frozen and lysed in buffer [1% Triton X-100, 1 mM EDTA in 1 x PBS (pH 7.4)] containing 10 µM leupeptin and 200 µM phenylmethylsulfonyl fluoride. The lysates were sonicated and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were collected, and the protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s protocol. Total protein (30 µg) was applied to each lane on 10% SDS-polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated with the following primary antibodies: anti-phospho-Raf-1(Ser338), anti-Raf-1, anti-phospho-MEK1/2(Thr202/204), anti-MEK1/2, anti-phospho-ERK1/2(Ser32/33), anti-ERK1/2, anti-phospho-p90RSK(Ser383), anti-p90RSK, anti-phospho-Bad(Ser112), anti-Bad, anti-cleaved PARP, and anti-α tubulin (diluted 1:1,000, Cell Signaling Technology, Beverly, MA, U.S.A.). The membrane was then incubated with secondary antibody (1:5000, Pierce, Rockford, IL, U.S.A.), and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) was used for detection according to the manufacturer’s protocol. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.).

Data analysis: All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student’s t-test. The difference for comparison was considered significant at * P<0.05.

RESULTS

We previously showed that melatonin significantly reduces infarct volume and apoptotic cell death [8, 9]. Figure 1A confirmed that melatonin significantly reduces apoptotic cell death caused by MCAO. The number of TUNEL positive cells significantly increased in the infarct region of vehicle-treated animals, whereas the number of TUNEL positive cells significantly decreased in melatonin-treated animals. The proportion of TUNEL positive cells was 73.6 ± 2.9% and 9.3 ± 2.1% in the ipsilateral cortex of vehicle- and melatonin-treated animals, respectively.

The phosphorylation of Raf-1, MEK1/2, and ERK1/2 were investigated to elucidate the neuroprotective signal pathway of melatonin in ischemic brain injury. Brain injury induced a reduction in phospho-Raf-1 level, while melatonin treatment prevented injury-induced down-regulation of phospho-Raf-1. The level of phospho-Raf-1 was 0.61 ± 0.02 in the ipsilateral cortex of vehicle-treated animals, whereas it was 0.95 ± 0.02 in melatonin-treated animals (Fig. 1B). Also, melatonin prevented the injury-induced a decrease of phospho-MEK1/2 and phospho-ERK 1/2. The level of phospho-MEK1/2 was 0.41 ± 0.03 and 0.83 ± 0.02 in the ipsilateral cortex of vehicle- and melatonin-treated animals, respectively (Fig. 2A). The level of phospho-ERK 1/2 was 0.62 ± 0.03 in the ipsilateral cortex of vehicle-treated animals, whereas it was 0.98 ± 0.02 in melatonin-treated animals (Fig. 2B). Brain injury induced a reduction in the phospho-p90RSK and phospho-Bad levels, whereas melatonin prevented injury-induced down-regulation of phospho-p90RSK and phospho-Bad. The level of phospho-p90RSK was 0.42 ± 0.02 in the ipsilateral cortex of vehicle-treated animals, whereas it was 0.75 ± 0.01 in melatonin-treated animals (Fig. 3A). The level of phospho-Bad was 0.73 ± 0.02 and 1.08 ± 0.04 in the ipsilateral cortex of vehicle- and melatonin-treated animals, respectively (Fig. 3B). In addition, we investigated the cleaved-PARP levels. We confirmed that brain injury induced an increase in cleaved-PARP levels and that melatonin prevented injury-induced cleavage of PARP. The level of cleaved-PARP was 1.18 ± 0.04 in the ipsilateral cortex of vehicle-treated animals, whereas it was 0.58 ± 0.03 in melatonin-treated animals (Fig. 4).
DISCUSSION

Melatonin prevents neuronal cell death from ischemic injury induced by MCAO [3]. Our previous study and others have shown that melatonin significantly reduces the infarct size and decreases apoptotic cell death from MCAO-induced damage [6, 8, 9, 16]. The present study confirms that melatonin significantly decreases the number of apoptotic cells in ischemic brain injury. Although it has been reported that melatonin prevents cytochrome c release from mitochondria and inhibits caspase-3 activation after cerebral ischemia [1, 5], the signal transduction pathways of melatonin during brain ischemia remained unknown. This study focused on the neuroprotective effect of melatonin on the activation of ERK 1/2 and its up- and down-stream targets, Raf, MEK, and p90RSK.

The MAP kinase signal pathway regulates the suppression of cell death and promotion of cell growth, differentiation, and survival [2, 15, 20]. Several growth factors initiate the protein kinase cascade that sequentially activates Raf-MEK-ERK-p90RSK. Previous studies have shown that ERK1/2 is activated by phosphorylation in response to melatonin, leading to attenuation of neuronal cell damage by ischemic injury [7]. Also, melatonin increases ERK1/2 phosphorylation levels in neuronal cell cultures [18]. This study demonstrated that the levels of ERK1/2 up-stream enzymes, phospho-Raf-1 and phospho-MEK1/2, decrease in cases of brain injury, and melatonin prevented the dephos-
phosphorylation of these kinases. Furthermore, melatonin prevents injury-induced decline of phospho-ERK1/2.

The MAP kinase pathway can prevent apoptotic cell death through the phosphorylation of the pro-apoptotic protein Bad. Phosphorylated p90RSK phosphorylates Bad at the Ser112 residue, which inactivates the pro-apoptotic function of Bad [24]. Bad is an apoptosis-related protein belonging to the BH3-only subfamily in the Bcl-2 family and promotes apoptosis [22]. Apoptotic stimuli dephosphorylate Bad and initiate the apoptotic cascade. However, phosphorylated Bad binds to 14–3–3, which inhibits Bad from interacting with Bcl-xL at the mitochondrial membrane. The phosphorylation of Bad is a critical event for the inhibition of apoptosis. In the absence of melatonin, the levels of phospho-p90RSK and phospho-Bad decrease in the injured region. Melatonin prevents injury-induced declines of phospho-p90RSK and phospho-Bad. Recently, we reported the fact that melatonin attenuates the focal cerebral ischemic injury by inhibiting the dissociation of phospho-Bad from 14–3–3 [8]. The binding of 14–3–3 and phospho-Bad inhibits the release of the pro-apoptotic protein Bax. Consequently, the maintenance of phospho-Bad and 14–3–3 interactions by melatonin prevents the activation of caspases-3, thus blocking activation of the apoptotic pathway [8]. Also, we previously showed that melatonin prevents neuronal cell death against ischemic brain injury through the activation of Akt signaling pathway [8, 9]. Activated Akt helps to stabilize the mitochondrial permeability transition (MPT) pore formation through the phosphorylation of Bad [23]. The activation of MPT pore formation indicates the release of cytochrome c from mitochondria into the cytosol and the activation of apoptosis. Finally, this study confirms that melatonin significantly prevents injury-induced cleavage of PARP and apoptotic cell death. In conclusion, the current study demonstrated that melatonin has a neuroprotective effect in ischemic brain injury and that this effect is mediated by the activation of MEK/ERK/p90RSK/Bad cascade signaling.

ACKNOWLEDGEMENTS. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006–311-E00519) and partially supported by (KRF-2007–313-E00538).

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