Melatonin Regulates Nitric Oxide Synthase Expression in Ischemic Brain Injury

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

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ABSTRACT. Nitric oxide (NO) is produced by three NO synthases (NOS), iNOS, eNOS, and nNOS. Production of NO by iNOS plays key roles in neurodegeneration, while eNOS is a protective enzyme. This study investigated the neuroprotective effect of melatonin and the levels of NOS isoforms induced by melatonin in ischemic brain injury. Adult male rats were treated with melatonin (5 mg/kg) or vehicle prior to middle cerebral artery occlusion (MCAO). Brains samples were collected at 24 hr after the onset of occlusion. Results confirmed that melatonin significantly reduces infarct area. Western blot analysis was used to evaluate the expression levels of iNOS, eNOS, and nNOS. The level of iNOS and nNOS increased in vehicle-treated animals, while melatonin prevented injury-induced increase of iNOS. In contrast to iNOS levels, eNOS levels decreased in vehicle-treated animals, while melatonin prevented the injury-induced decrease of eNOS. This study provides further evidence that melatonin exerts neuroprotective effects, and the regulation of NOS isoforms by melatonin may contribute to the neuroprotective effects.

KEY WORDS: melatonin, neuroprotection, NOS.

Melatonin has a number of physiological functions, including the regulation of circadian rhythms, the inhibition of the oxidation of biomolecules, and the removal of free radicals [14, 20]. Moreover, melatonin plays a neuroprotective role against transient or permanent ischemic brain injury [8, 10, 16]. In clinical studies, a decrease of melatonin is related to neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease [3, 18].

Nitric oxide (NO) is a critical player in pathological and physiological processes in the central nervous system [19, 22]. NO is generated by the activation of three distinct nitric oxide synthases (NOS), inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). NO exerts both neuroprotective and neurotoxic effects in focal cerebral ischemia [4, 17]. iNOS produces NO with cytotoxic effects, whereas eNOS plays a protective role. Furthermore, melatonin has antioxidant activity and can inhibit free radical formation thereby decreasing NOS [2, 12]. Although previous studies have elucidated the neuroprotective mechanism of melatonin, little data is available regarding the levels of NOS isoforms. Thus, this study investigated the neuroprotective effect of melatonin and its effect on the expression of NOS isoforms after ischemic brain injury.

All 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. Male Sprague-Dawley rats (230-250 g, n=30) were used in this study. Animals were maintained under controlled temperature (25°C) and lighting (12/12 light/dark cycle) and allowed to have free access to food and water. Animals were randomly divided into two groups, vehicle-treated group and melatonin-treated group (n=15 per group). All experiments were performed during the day between 10:00 and 16:00 hr. Melatonin (Sigma Chemical, St. Louis, MO, U.S.A.) was dissolved in normal saline (1 mL) containing less than 5% dimethyl sulfoxide (Sigma Chemical) 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 (MCAO) [16].

MCAO was induced as previously described [13]. Before surgery, animals were anesthetized with sodium pentobarbital (100 mg/kg). Briefly, 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 gentle heating, was inserted through the right internal carotid artery to the base of the middle cerebral artery. At 24 hr after the onset of permanent occlusion, animals were decapitated, and the brains were rapidly removed. Brains were cut into coronal slices of 2 mm in thickness, and these slices were reacted with a 2% triphenyltetrazolium chloride (TTC; Sigma) for 20 min to reveal the ischemic infarction. After the TTC reaction, these slices were fixed with 10% formalin solution. The stained slices were photographed by a Nikon CoolPIX990 digital camera (Nikon, Tokyo, Japan) and measured for the ischemic lesion by Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, U.S.A.). The ischemic lesion percentage of each slice was calculated by the ratio of the infarction area to the whole slice area.

The brains were dissected into ipsilateral and contralateral cortices, quickly lysed in buffer [1% Triton X-100, 1 mM EDTA in 1 × PBS (pH 7.4)] containing 10 µM leupeptin and 200 nM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 15,000 g for 20 min at 4°C. The supernatants were collected and the protein concentration of each lysate was determined using the bicinchoninic acid (BCA)
of others have shown that melatonin decreases infarct size in MCAO [8]. Our previous study as well as the work of others have shown that melatonin decreases infarct size and apoptotic cell death from MCAO-induced damage [9, 11, 16]. This study confirmed the neuroprotective effect of melatonin in ischemic brain injury using TTC staining. In addition, our previous study demonstrated that melatonin mediates its neuroprotective effects through the inhibition of apoptotic signaling and the activation of Akt pathway [11].

Ischemic damage is induced by the accelerated formation of reactive oxygen species including superoxide and NO radicals [21]. NO is produced by three NOS isoforms. Among the three NOS isoforms, iNOS is expressed by activated microglia and astrocytes after focal brain ischemia [7]. iNOS contributes to NO synthesis, increasing free radical formation and promoting brain damage after stroke [5]. Our results showed that iNOS levels increase in cases of brain injury and that melatonin prevents increase of the iNOS levels. Also, a previous study has demonstrated that tissue-plasminogen activator-induced brain injury increases iNOS levels, while melatonin decreases the accumulation of iNOS [10]. Furthermore, inactivation of iNOS and knocking out the iNOS gene reduce brain damage in ischemic brain injury [6]. This study showed that melatonin decreases the expression of iNOS and prevents cell death during the MCAO-induced brain injury.

Both nNOS and iNOS play prominent roles in neurodegeneration, while eNOS prevents neuronal injury. Concretely, the overproduction of NO from nNOS or iNOS leads to neurotoxicity, while NO production from eNOS prevents brain damage by maintaining regional cerebral blood flow [17]. Levels of nNOS increase in MCAO-induced brain injury, and melatonin prevents increase of the nNOS levels. Melatonin inhibits nNOS activity through its binding to the calcium-calmodulin complex [12]. Previous studies demonstrate that eNOS is an essential factor for endothelial function and is a major protective factor in the pathophysiology of vascular endothelium [1, 15]. The reduction of eNOS expression leads to extensive damage by platelet aggregation and neutrophil infiltration [6]. Furthermore, the up-regulation of eNOS contributes to the protective mechanism of the endothelium by increasing blood flow [1]. Our results showed that eNOS levels decline in MCAO-induced brain injury, and melatonin prevents decrease of eNOS levels. Melatonin provides a significant microvascular protection through the activation of eNOS [23]. Moreover, melatonin-induced protection involves vascular mechanism through the vasodilatation after cerebral ischemia [9]. Melatonin represses up-regulation of iNOS and nNOS, and the down-regulation of eNOS in MCAO-induced brain injury. Taken together, our results suggest that melatonin prevents cell death caused by ischemic brain injury. Also, the neuroprotective effects of melatonin are mediated through the inhibition of iNOS and nNOS and activation of eNOS. However, future studies are required in order to elucidate the mechanism by which melatonin regulates NOS isoforms. This study supports the hypothesis that melatonin protects neuronal cells in ischemic brain injury and that regulation of NOS isoforms by melatonin contrib-
Fig. 1. Representative photos of triphenyltetrazolium (A) staining and Western blot analysis of iNOS (B), nNOS (C) and eNOS (D) in rat brain. Rats were treated with vehicle (labeled C) or melatonin (labeled M) prior to MCAO. A: TTC showed that the ischemic area remained white, while the intact area was stained red. Melatonin treatment significantly protected the cerebral cortex from ischemic brain injury. B-D: Western blot analysis was performed in the ipsilateral (IPSI) cortex and contralateral (CONTRA) cortex. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. Data (n=15) are represented as mean ± S.E.M.; * P<0.01, ** P<0.05.

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REFERENCES

4. Dalkara, T. and Moskowitz, M.A. 1994. Brain Pathol. 4: 49-