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EFFECTS OF MEMANTINE AND MELATONIN ON SIGNAL TRANSDUCTION PATHWAYS VASCULAR LEAKAGE AND BRAIN INJURY AFTER FOCAL CEREBRAL ISCHEMIA IN MICE

U. KILIC,[a] B. YILMAZ,[b] R. J. REITER,[c] A. YÜKSEL[a] AND E. KILIC[b,d]*

[a] *Bezmialem Vakif University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey*

[b] *Yeditepe University, Faculty of Medicine, Department of Physiology, Istanbul, Turkey*

[c] *Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA*

[d] *Istanbul Medipol University, Faculty of Medicine, Department of Physiology, Istanbul, Turkey*

Abstract—Because of their favorable action profiles in humans, both memantine and melatonin are particularly interesting candidates as neuroprotectants in acute ischemic stroke. Until now, the signaling mechanisms mediating memantine's neuroprotective actions remained essentially uninvestigated. In addition, we have combined memantine with melatonin, which is a well-known neuroprotective molecule. Herein, we examined the effects of memantine (20 mg/kg, *i.p.*) administered alone or in combination with melatonin (4 mg/kg, *i.p.*) on the activation of signaling transduction pathways, IgG extravasation and ischemic injury in mice submitted to 90 min of intraluminal middle cerebral artery occlusion, followed by 24 h of reperfusion. In these studies, both agents reduced ischemic injury and the density of DNA-fragmentation. Notably, melatonin/memantine combination reduced ischemic injury further as compared with memantine treatment, which was associated with reduced IgG extravasation, indicating vascular leakage in the brain. Animals receiving memantine exhibited elevated ERK-1/2 and decreased p21 and p38/MAPK activations, while it had no significant effect on phosphorylated Akt and SAPK/JNK1/2 in the ischemic brain. However, melatonin increased the activation of Akt and reduced the activations of ERK-1/2, p21, p38/MAPK and SAPK/JNK1/2 significantly. Synergistic effects of memantine and melatonin were observed in the inactivation of p21, p38/MAPK and SAPK/JNK1/2 pathways. Moreover, memantine reversed the effects of melatonin on the activation of ERK-1/2 pathway. Here, we provide evidence that free radical scavenger melatonin potentiates the effects of memantine on ischemic brain injury via inactivations of p21 and stress kinases p38/MAPK and SAPK/JNK1/2 pathways.

Key words: melatonin, memantine, cerebral ischemia, cell signaling, blood–brain barrier permeability.

INTRODUCTION

Brain injury following ischemic stroke develops from a complex series of pathophysiological events, including over-activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (excitotoxicity), disruption of cellular Ca^{2+} homeostasis and free radical formation (Dirnagl et al., 1999; Reiter et al., 2005). In aspect of the complex pathology of ischemic stroke and the disappointing outcome of many clinical trials, we hypothesized that combination of neuroprotective compounds, targeting different events observed in the ischemic brain tissue, may be more promising in patients than using a single drug.

NMDA receptors (NMDAR) are mainly found on the postsynaptic membranes at the synaptic and also extrasynaptic positions (Léveillé et al., 2008). Activation of NMDA receptors plays crucial roles in the development of central nervous system, synaptic plasticity, learning and memory formation (Suzuki et al., 2006; Wang and Qin, 2010). However, when NMDA receptors become over-activated excessive amounts of Ca^{2+} are accumulated in the cytoplasm and mitochondria (Lipton, 2004). Ca^{2+} overload activates enzymes such as phospholipase A_2 and cyclooxygenase leading to free-radical generation, which overwhelms the innate free-radical scavenging mechanisms (Dirnagl et al., 1999; Lipton, 2004). In addition to Ca^{2+} overload, activated enzymes and free radicals stimulate neuronal nitric oxide synthase (nNOS) leading to the generation of nitric oxide (NO) and highly toxic peroxynitrite ($ONOO^-$). Thereafter, phosphorylations of ERK-1/2 and stress kinases, p38/MAPK and SAPK/JNK1/2, activate pro-apoptotic transcription factors and immediate early genes mediating inflammation and apoptosis (Dirnagl et al., 1999; Lipton, 2004; Martel et al., 2009; Wang and Qin, 2010; Choo et al., 2012; Kaufman et al., 2012).

Memantine is a non-competitive NMDA receptor blocker with fast channel unblocking kinetics which prevents it from occupying the channels and interfering

*Correspondence to: E. Kilic, Istanbul Medipol University, Faculty of Medicine, Department of Physiology, TR-34083 Istanbul, Turkey. Tel: +90-212-4448544; fax: +90-212-531-7555.
E-mail address: kilic44@yahoo.com (E. Kilic).

Abbreviations: CBF, cerebral blood flow; LDF, laser Doppler flow; MCA, middle cerebral artery; MCAO, MCA occlusion; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; ROI, regions of interest; TBS-T, Tris-buffered saline containing 0.1% Tween.

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with normal synaptic transmission (Volbracht et al., 2006; Wroge et al., 2012) via preventing excessive NMDA receptor activity particularly at the extrasynaptic position (Lipton and Chen, 2004; Wroge et al., 2012). It has been previously shown that memantine protects hippocampal cells from hypoxic injury *in vitro* (Wroge et al., 2012) and *in vivo* (Volbracht et al., 2006; Liu et al., 2009). Moreover, memantine prevents memory impairment (Watanabe et al., 2010) and decreases ischemic injury and neurological deficit after cerebral ischemia, when administered prior to ischemia (Babu and Ramanathan, 2011). However, signaling pathways via which memantine protects the brain against ischemia remained largely unknown after focal cerebral ischemia.

Melatonin is a powerful free radical scavenger with the desirable characteristics of a clinically-reliable antioxidant which detoxifies oxygen- and nitrogen-based free radicals, including the highly toxic hydroxyl- and $ONOO^-$-radicals (Reiter et al., 2010; Galano et al., 2011). In addition to its antioxidant effect, electrophysiological studies showed that melatonin depresses NMDAR mediated activity in the brain (León et al., 1998; El-sherif et al., 2002), and it was suggested that melatonin inhibits NMDA-induced excitation by reducing nNOS activity, and thus NO production, and by an interaction of melatonin with the redox site of the NMDAR (Escames et al., 2004). Moreover, it has been shown that melatonin prevents glutamate-mediated LTP induction via the inhibition of phosphorylation of CaM kinase II, without changes in basal synaptic transmission (Fukunaga et al., 2002). In this context, recent studies showed that melatonin protects the brain against ischemic stroke in rats and mice (Kilic et al., 1999; Tai et al., 2010). Furthermore, it has been demonstrated that melatonin treatment inhibited time-dependent elevation of beta-amyloid, reduced abnormal nitration of proteins, and increased survival in mice (Matsubara et al., 2003). In fact, melatonin decreases infarct size (Kilic et al., 2012), reduces DNA fragmentation, caspase-3 activity (Kilic et al., 2004a) and diminishes mitochondrial cytochrome c release (Andrabi et al., 2004).

To investigate the effects of memantine, melatonin and their combination in focal brain ischemia, we conducted the present experiments in which we submitted C57/BL6 mice to 90 min of middle cerebral artery (MCA) thread occlusions. Twenty-four hours after reperfusion, we analyzed activations of Akt, ERK-1/2, p21 and stress kinases, p38/MAPK and SAPK/JNK1/2 signaling, as well as, ischemic injury, DNA fragmentation, and IgG extravasations.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed in accordance to National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and approved by local government authorities (Yeditepe University, Animal Research Ethics Committee). All animals were maintained under a constant 12:12-h light–darkness regimen (lights on daily at 07.00 h). In this study, adult male C57BL/6j mice weighing 22–26 g were randomly assigned to one of four groups and subjected to focal cerebral ischemia. The animals were treated with an intraperitoneal injection of (i) vehicle (100 μL isotonic saline containing 5% ethanol; $n = 8$); (ii) melatonin (4 mg/kg; $n = 8$); (iii) memantine (20 mg/kg; $n = 8$) and (iv) melatonin combined with memantine ($n = 8$) immediately after reperfusion. The drug treatments were repeated 20 min after reperfusion onset with melatonin (2 mg/kg) and memantine (10 mg/kg) alone or in combination.

MCA occlusion (MCAO)

Animals were anesthetized with 1% isofluorane (30% O_2 remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. During the experiments, cerebral blood flow (CBF) was monitored by laser Doppler flow (LDF) recordings using a flexible 0.5-mm fiber optic probe (Perimed, Stockholm, Sweden), which was attached to the intact skull overlying the MCA territory (2 mm posterior/6 mm lateral from bregma). LDF changes were monitored up to 30 min after the onset of reperfusion. Focal cerebral ischemia was induced using an intraluminal filament technique (Kilic et al., 2010). A midline neck incision was made, and the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691; Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 8–0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren; Bayer Dental, Osaka, Japan; diameter of the coated thread: 180–190 μm) was introduced through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for MCAO. Ninety minutes after induction of ischemia, reperfusion was initiated by withdrawal of the monofilament. Thirty minutes after reperfusion onset, wounds were closed with suture, before anesthesia was discontinued.

Twenty-four hours after MCAO, animals were deeply anesthetized with 4% isofluorane (30% O_2, remainder N_2O) and decapitated. Brains were removed, frozen on dry ice, and cut on a cryostat into coronal 18-μm sections that were used for studying infarct volume and immunohistochemistry studies. From the same animals, tissue samples were taken from the MCA stroke territory (striatum and overlying parietal cortex) for protein analysis by Western blot.

Analysis of ischemic injury

Coronal brain sections from four equidistant brain levels, 2 mm apart, were stained with Cresyl Violet according to a standard protocol. On the sections, the border between infarcted and non-infarcted tissues was outlined using an image analysis system (Image J; National Institute of Health, Bethesda, MD, USA), and the area of infarction was assessed by subtracting the area of the non-lesioned ipsilateral hemisphere from that of the contralateral side. The volume of infarction was calculated by integration of these lesion areas.

Evaluation of serum IgG extravasation

With gentle stirring, brain sections obtained from the mid striatum were rinsed for 10 min at room temperature in 0.1 M phosphate-buffered saline (PBS), to remove intravascular IgG, and were fixed in 4% paraformaldehyde. After blocking of endogenous peroxidase with methanol/0.3% H_2O_2 and immersion in 0.1 M PBS containing 5% bovine serum albumin and normal swine serum (1:1000), sections were incubated for 1 h in biotinylated goat anti-mouse IgG (1:200; Santa Cruz) and stained with avidin peroxidase kit (Vectastain Elite; Vector Labs,