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Protective effect of melatonin against transient global cerebral ischemia-induced neuronal cell damage via inhibition of matrix metalloproteinase-9

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ABSTRACT

Aims: Melatonin possesses various pharmacological effects including neuroprotective effects against brain ischemia. Post-ischemic increases in matrix metalloproteinase-9 (MMP-9) expression and activity mainly contribute to neuronal damage by degradation of the extracellular matrix. This study was designed to examine whether melatonin has a neuroprotective effect and an influence on MMP-9 in transient global brain ischemia.
Main methods: Mice were subjected to 20 min of global brain ischemia and sacrificed 72 h later. Melatonin (30 mg/kg) was administered 30 min before and 2 h after ischemia as well as once daily until sacrifice.
Key findings: Hippocampal pyramidal cell damage after ischemia was significantly decreased by melatonin. As observed by zymography, melatonin inhibited the increase of MMP-9 activity after ischemia. In the brain sections, the increased gelatinase activity was mainly observed in the hippocampus after ischemia and melatonin also reduced gelatinase activity. The laminin and NeuN expression levels were reduced in the hippocampal CA1 and CA2 regions after ischemia, and melatonin reduced laminin degradation and neuronal loss. A TUNEL assay demonstrated that there were TUNEL-positive cells in the hippocampus and the number of TUNEL-positive cells was significantly decreased by melatonin. There was no difference in the ischemia-induced hippocampal neuronal damage between the vehicle- and melatonin-treated groups of MMP-9 knock-out mice.
Significance: These data demonstrate that melatonin suppressed the occurrence of neuronal injury, which might be partly due to its inhibitory effects on MMP-9 in addition to its anti-oxidative effects. MMP-9 may be an important key target of melatonin in neuroprotection against global ischemia.

Introduction

In transient global cerebral ischemia, selective neuronal injury occurs in the brain tissue that is vulnerable to the brain injury. This type of cellular injury leads to delayed neuronal injury whose morphologic features become notable after a considerable length of time (Pulsinelli et al., 1982). Delayed neuronal injury mainly occurs in the hippocampal areas which are vulnerable to global ischemia. During ischemia, glutamate is abnormally released from the synaptic area. Following the synapse, the excessive calcium influx into the neuron is induced by the glutamate receptor with the calcium channel at the terminal end of the neuron (Choi, 1990). This leads to the activation of adverse proteases. In addition, there is also excitatory neurotoxicity where the cellular injury occurs due to proteolysis by the neurons themselves (Illievich et al., 1994). Furthermore, the cells are structurally damaged, which includes damage to the plasma membrane caused by the free radicals that are excessively increased during ischemia. Apoptosis is also a major pathophysiological process involved in the cellular injury following brain ischemia (Bondy, 1995; Chan et al., 1998; Chan, 2001; Lipton, 1999).

Moreover, matrix metalloproteinase (MMP), one of the enzymes involved in the degradation of the extracellular matrix inhibits the interactions between the neurons and the extracellular matrix by degrading the extracellular matrix (Rosenberg, 2002), which in turn induces anoikis-like cell death (Gu et al., 2002). Among more than 20 types of MMPs, MMP-2 and MMP-9 are closely associated with the pathogenesis of cerebral ischemia (Lo et al., 2002). MMP-9 might especially be involved in the destruction of the blood–brain-barrier, cerebral edema, cerebral hemorrhage, neuroinflammation, and neuronal damage (Gu et al., 2002; Lo et al., 2002; Rosenberg, 2002). MMP-9 knockout mice are reported to have reduced brain damages after ischemia compared to wild-type mice (Asahi et al., 2001; Lee et al., 2004).

Melatonin has various pharmacological actions including an antioxidant effect, an anti-aging effect, an immune-strengthening effect, and a neuroprotective effect (Reiter, 1991). In addition, melatonin has also been known to suppress brain injury due to cerebral ischemia

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(Cervantes et al., 2008; Pei et al., 2002; Rennie et al., 2008). According to other literatures, melatonin has an inhibitory effect on MMP in various tissues (Ganguly et al., 2005; Esposito et al., 2008; Hung et al., 2008; Mao et al., 2010). Recently, in particular, it was found that melatonin binds to MMP-9 (Rudra et al., in press). The report also showed that melatonin suppresses MMP-9 activity. Based on the fact that MMP has a significant effect on the pathogenesis of ischemic neuronal injury, melatonin might have protective effect against ischemia. Given the aforementioned background material, we examined whether melatonin has an inhibitory effect on the occurrence of delayed neuronal injury in a murine experimental model of transient global ischemia. We also examined whether these effects are based on the inhibitory effects of MMP.

Materials and methods

Experimental animals and surgery to induce global ischemia

All procedures in this study were approved by the Keimyung University School of Medicine Animal Care and Use Committee. In the current study, we used male C57BL/6 mice weighing 22–27 g. To study the influences of MMP-9, we used MMP-9 knock-out (KO) mice (FVB(Cg)-*Mmp9*^{tm1Tvu}/J, Jackson Lab., USA) and C57BL/6 J for the MMP-9 wild-type (WT) as matched controls. Animals were allowed to freely ingest water and foods under a 12:12 light-dark cycle. The room temperature was maintained at 22 ± 1 °C. To induce global cerebral ischemia, surgery was performed under isoflurane anesthesia (3% for induction anesthesia and 1.5% for maintenance anesthesia) with N_2O (70%) and O_2 (30%). With the dissection of the neck skin, the bilateral common carotid arteries were exposed and then carefully isolated from the adjacent nerve and tissue. The bilateral common carotid arteries were occluded for 20 min using microclips to induce global cerebral ischemia, which was followed by reperfusion. To prevent the occurrence of hypothermia during surgery, the rectal temperature was maintained at 37 ± 0.5 °C using a feedback thermoregulator (CMA 150, CMA Microdialysis, Sweden). Our experimental animals achieved recovery in a box whose temperature was postoperatively maintained at 30 °C for at least 3 h. Seventy-two hours following the induction of ischemia, experimental animals were sacrificed for further experimental procedures.

The measurement of the cerebral perfusion

Using a Laser Doppler flowmeter (5000 system, Perimed, Sweden), the degree of cerebral blood flow (CBF) was measured in the parietal area of the experimental animals. We included only the experimental animals whose CBF was decreased by less than 15% following the occlusion of the bilateral common carotid arteries compared with prior to occlusion.

The administration of the drugs

Melatonin (Sigma, Korea) was dissolved in a 5% ethanol. Then, at a dose of 30 mg/kg, it was intra-abdominally injected 20 min prior to the induction of ischemia and 2 h after induction. This was followed by once-daily injections until the experimental animals were sacrificed for the experimental procedure. For the control group, our experimental animals were given the same volume of 5% ethanol as the mice that received treatment, according to the same schedule.

SDS-PAGE Gelatin gel zymography

To examine gelatinase, MMP-9 and MMP-2 activities, gelatin gel zymography was performed. After 72 h of reperfusion, mice were anesthetized deeply with ethyl ether and then perfused transcardially with ice-cold PBS (pH 7.4). Their brains were quickly removed and the hippocampi were dissected, frozen immediately under ice-cold conditions and stored at −75 °C. Using the method described by Asahi et al. (2001), the hippocampi were homogenized in lysis buffer with protease inhibitors. After centrifugation, the supernatant was collected and the total protein concentration was determined using the Bradford assay (Bio-Rad, USA). Forty-microgram protein samples were mixed with 2× sample buffer and separated by 10% Tris-glycine gel using 0.1% gelatin as the substrate. After electrophoresis, the gel was renaturated and then incubated with developing buffer at 37 °C for 24 h. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min and then destained appropriately. The pale-colored bands of proteolysis observed by zymography were quantified using scanning densitometry (Quantity One, Bio-Rad).

Histological examination

Seventy-two hours following the induction of ischemia, our experimental animals were deeply anesthetized using ether. After opening the thorax, we perfused a phosphate-buffered saline (PBS, pH 7.2) using a syringe through the left ventricle. The brain was extracted from the skull and then frozen immediately. Then, the brain was sectioned at a thickness of 14 μm using a cryostat. Coronal sections of the brain were fixed in a 10% formalin solution for approximately an hour and then stained with H & E dyes. Both the CA 1 and 2 regions are the most vulnerable to ischemia in C57BL/6 mice (Yang et al., 1997). We therefore examined the degree of damages to the neurons in both regions, for which we measured the more severely damaged side when comparing the left and right side. With the modified methods described by Tsuchiya et al. (Tsuchiya et al., 2002), we semi-quantified the number of damaged cells based on a 4-point scale: 0 points (no ischemic injury to the pyramidal neurons in the CA1 and CA2 regions), 1 point (ischemic injury to fewer than 20% of the pyramidal neurons in the CA1 and CA2 regions), 2 points (ischemic injury to 20–50% of the pyramidal neurons in the CA1 and CA2 regions), 3 points (ischemic injury to more than 50% of pyramidal neurons in the CA1 and CA2 regions) and 4 points (ischemic injury to the overall areas in the CA1 and CA2 regions and all of the other remaining areas of the hippocampus).

In situ zymography

In situ zymography cannot be used to differentiate between the activity of MMP-9 and MMP-2 in the tissue samples. However, in situ zymography is used to determine the activity and location of the enzymes (Lee et al., 2004). Following reperfusion with PBS through the heart, the brain tissue was not fixed but was instead promptly removed. Then, the tissue was rapidly frozen with 2-methylbutane and liquid nitrogen. The tissue samples were sectioned using a cryostat at a thickness of 14 μm, followed by reaction with Enz-Check kit (Molecular Probe, USA) at 37 °C for approximately 18 h, allowing for fluorescent microscopy. Thus, we attempted to detect the activity of gelatinase by fluorescent microscopy after the samples were exposed to the fluorescein isothiocyanate (FITC) signals, wherein the FITC-conjugated gelatin is destroyed with the help of enzymes. This technique is useful for determining the activity of gelatinase distributed in each hippocampal region. To measure the relative intensity of the FITC fluorescent signal, the medial portion of the CA1 and total CA2 areas in the hippocampus were examined because both regions were sensitive to transient global cerebral ischemia in our model. The analysis was performed according to the previously reported method (Lee et al., 2004). In brief, images of in situ zymography were captured by a Leica DM 3000 microscope and CCD camera (Leica DFC 480). Quantification was performed using image analysis software (Carl Zeiss LSM Image Examiner version 4.2.0.121). The fluorescent signal was measured by a blind examiner and presented as the percentage of FITC fluorescence intensity of the in situ zymography (Hong et al., 2012).