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Two different molecular mechanisms underlying progesterone neuroprotection against ischemic brain damage

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ABSTRACT

Herein, we show that a single injection of P4 (4 mg/kg) at 1 h or 48 h, but not 96 h, before middle cerebral artery occlusion (MCAO) produces significant protective effects against the ischemia-induced neuronal death and the deficits in spatial cognition and LTP induction. The present study focused on the molecular mechanisms underlying the neuroprotection exerted by P4 administration at 1 h and 48 h pre-MCAO, termed acute and delayed P4-neuroprotection, respectively. Pharmacology suggested that P4-receptor (P4R) cascading to a Src-ERK1/2 signaling mediated the delayed P4-neuroprotection. To support this, it was observed by anti-phosph-ERK1/2 immunoblots that a single injection of P4 triggered a P4R-mediated persistent increase in ERK1/2 phosphorylation and their nuclear translocation for 48 h. In contrast, the acute P4-neuroprotection did not depend on the P4R-mediated Src-ERK1/2 signaling. Instead, the acute P4-administration attenuated the NMDA-induced rise in the intracellular calcium concentration ($[Ca^{2+}]_i$) that may be a primary cause for MCAO-induced neuronal injury. This effect seemed to be exerted by an antagonism of σ_1 receptor since the σ_1 receptor antagonist NE100 perfectly mimicked the acute P4-neuroprotection and also attenuated the NMDA-induced $[Ca^{2+}]_i$ increase. These findings suggest that the P4 neuroprotection involves two independent processes depending on the timing of P4 administration before MCAO: an acute protection by antagonizing σ_1 receptor to inhibit NMDAr-Ca^{2+} influx and a delayed one by an activation of P4R-mediated Src-ERK signaling pathway.

1. Introduction

Premenopausal women have lower risks of stroke (Sacco et al., 1997) and better outcomes following stroke (Thorvaldsen et al., 1995) relative to men of the same age. Following the menopause, the incidence of stroke in women rapidly increases (Hodis and Mack, 2007) coincident with decreased circulating levels of the sex steroid hormones estrogen (E2) and progesterone (P4) (Amantea et al., 2005). Whilst the majority of research has focused on E2 as the main source of neuroprotection seen in female animals, there is increasing evidence that P4 by itself exerts a potential neuroprotective effect on ischemia-induced brain injury (Chen et al., 1999; Kumon et al., 2000; Morali et al., 2005; Sayeed et al., 2006) and promotes functional recovery after cerebral ischemia (Gibson and Murphy, 2004; Sayeed et al., 2007). Importantly, when administered before or after cerebral ischemia and traumata P4 affords neuroprotection for preventing and treating stroke (Jiang et al., 1996; Murphy et al., 2002; Sayeed et al., 2007). However, the underlying mechanisms of the P4-neuroprotection are not completely understood to date.

P4 activates intracellular signaling pathways involved in the promotion of cell survival (Singh, 2005, 2006); it increases the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-XL and decreases the expression of pro-apoptotic molecules such as Bax, Bad and caspase-3 (Yao et al., 2005). Some of these effects of P4 may be mediated by the activation of progesterone receptor (P4R), a classical nuclear receptor (Guerra-Araiza et al., 2003). A large body of evidence emerges that a P4R-activated Src-ERK1/2 signaling pathway is involved in mammalian breast cancer cells (Ballare et al., 2003; Faivre et al., 2005). In addition, P4 has been identified as a potent modulator of the NMDA receptor (NMDAr) and $GABA_A$ receptor activities (Monnet et al., 1995; Majewska, 1992). Recently, much attention has been attracted to the regulative effects of P4 on the activity of sigma-1 (σ_1) receptor (Maurice et al., 2006; Monnet and Maurice, 2006). Activation of σ_1 receptor increases the NMDAr

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activity (Martina et al., 2007) to enhance the glutamate induced intracellular Ca^{2+} increases in rat hippocampal pyramidal neurons (Monnet et al., 2003). Those results on the P4 mediated intracellular signaling, however, have not been well utilized to explore the molecular mechanisms of P4-neuroprotection against ischemic brain damages.

Herein, we report that a single administration of P4 (4 mg/kg) at either 1 h or 48 h but not 96 h before cerebral ischemia produces a significant protective effects, in terms of reducing ischemia-induced neuronal death, deficits in spatial cognition and LTP induction. The present study further elucidated the underlying molecular mechanisms of the P4-neuroprotective effects. Our results determined that the P4 neuroprotection seems to involve two independent processes depending on the administration timing before MCAO: an acute protection by antagonizing σ_1 receptor to reduce NMDA-increased $[Ca^{2+}]_i$ and a delayed one by activating P4R-mediated Src-ERK1/2 signaling pathway. The former mechanism is the first demonstration that P4 exerts its neuroprotective effects by targeting σ_1 receptor.

2. Materials and methods

Male Sprague–Dawley rats (Oriental Bio Service Inc., Nanjing), weighing 200–250 g before experiments, were used throughout the study. The animals were housed in a light controlled room under a 12-h light-dark cycle starting at AM 7:00 and kept at a temperature of 25 °C. Animals were given unrestricted access to food and water. All procedures were in accordance with the guidelines of Institute for Laboratory Animal Research of the Nanjing Medical University.

2.1. Preparation of ischemia model

Anesthesia was induced by 2% halothane and maintained with 1% halothane in 70% N_2O and 30% O_2 using a vaporizer. The rectal temperature was controlled at 37 ± 5 °C with a homoeothermic blanket. After the midline skin incision, the right common carotid arteries was isolated and ligated proximally with a 6-0 silk suture. The external carotid artery, occipital artery, and pterigopalatine artery were isolated and cauterized. A poly-L-lysine (0.1%, weight volume^{-1})-coated nylon monofilament thread (3/0 gauge with the tip heat blunted to a diameter of 0.29 mm) was inserted into the external carotid artery and manipulated to enter the internal carotid artery, where it was advanced until resistance was felt (approximately 20 mm) at the point where the filament blocks the middle cerebral artery, with the use of the procedure described by Mulcahy et al. (2003). At 60 min after MCAO, the thread was withdrawn to permit reperfusion of the middle cerebral artery. The wound was closed and the animal returned to its cage. As we did not measure the extent of occlusion due to lack of proper instrument in our lab, the extent of occlusion can only be judged by the resulting ischemic injury. Neuronal injury caused by our method was very stable, indicating that the method used was very reproducible.

P4 was dissolved in dimethylsulfoxide (DMSO), then in saline solution, final vehicle being DMSO 5% in saline. P4 was administered by a single intraperitoneal injection at 1 h, 48 h or 96 h before MCAO at 4 mg/kg. We selected this low dosage because P4 at this dosage was reported to reduce significantly the ischemic damage and regulate anti-apoptotic gene expression following traumatic brain injury in rats (Murphy et al., 2002; Yao et al., 2005). To analyze the underlying molecular mechanisms of P4 actions, we used a variety of inhibitors, all of which were given 30 min before P4-administration. The P4R antagonist RU486 was administered by an intraperitoneal injection, while other inhibitors including U0126, PP2 and PP3 were infused into the cereboventricle with a stepper-motorized microsyringe (Stoelting, Wood Dale, IL) as described previously (Hou et al., 2003). Rats were anesthetized with 2% halothane, and 26-gauge stainless-steel needle was inserted stereotaxically into the left cereboventricle. The inhibitors were prepared freshly on the day of experiment (final volume = 5 μl/rat). Control rats were given an equal volume of saline.

2.2. Histological examination

Neuronal cell loss was assessed by histological examination of toluidine blue-stained brain sections at the level of the middle hippocampus from animals killed at 7 days after ischemia or sham operation. Animals were deeply anesthetized with pentobarbital (50 mg/kg); this was followed by transcardiac perfusion with ice-cold 4% paraformaldehyde in PBS [0.1 m (pH 7.4)]. Brains were removed and immersed in fixative (4 °C overnight), and then processed for paraffin embedding. Coronal sections (4 μm in thickness) were cut at the level of the hippocampus, and every fourth section was collected and stained with toluidine blue.

Under a light microscope, healthy CA1 pyramidal neurons showed a round cell body with a plainly stained nucleus (Fig. 1Aa–f). Density of surviving neurons was expressed as the number of cells per mm length measured along the hippocampal CA1 pyramidal layer counted under a light microscope at ×400 magnifications as described previously (Guan et al., 2006; Wang et al., 2006). Neuronal densities measured from four sections per animal were averaged to provide a single value for each animal. We also made supplemental examinations on several slices stained with trypan blue that stains dead cells, and obtained results that were essentially the same as that determined by eye with the toluidine blue stained slices. To exclude the possibility that alterations in cell density value might be a consequence of changes in the volume of reference, we used a subset of the sections for volume estimation of CA1 *stratum radiatum*. Areas of CA1 *stratum radiatum* were measured in each section using Scion Image software (Scion Corp., Frederick, MD).

2.3. Electrophysiological analysis

On 7th day post-MCAO, rats were decapitated under deep anesthesia with ethyl ether. The brain was taken quickly, and placed in ice-cold artificial cerebrospinal fluid (ACSF) for about 10 min. ACSF (in mM: NaCl 126, KCl 2.7, KH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10) was oxygenated with a gas mixture of 95% O_2 and 5% CO_2 and the pH was adjusted to 7.4. Coronal slices (400 μm) were cut using a vibrating microtone (Microslicer DTK 1500, Dousaka EM Co, Kyoto, Japan). After a slice was transferred to a submerged recording chamber, constant current pulses (100 μs, 0.05 Hz) were supplied by a bipolar tungsten electrode using a stimulator (SEN-3301, Nihon Kohden, Japan) to stimulate the Schaffer collateral/commissural pathway. Excitatory postsynaptic potential (EPSP) was recorded extracellularly from the *stratum radiatum* with a 4–5 MΩ resistance glass microelectrode connected to a neutralized, high input-impedance preamplifier with a low-cut filter (5 kHz). The EPSPs were then digitized using the pCLAMP system (Axon Instrument Inc., CA). The intensities of the test pulse and the high-frequency stimulation (HFS, 100 Hz, 1 s) for LTP induction were adjusted to evoke 50% of the maximum EPSP response and 40% of the maximum EPSP response, respectively, in each slice from sham-op and MCAO rats.

2.4. Behavioral analysis

For the "Morris" water maze task, a pool (180 cm in diameter) was prepared with black plastic, and the water temperature was maintained at 20 °C. Swimming paths were analyzed by a computer system with a video camera (AXIS-90 Target/2; Neuroscience). In the hidden-platform test, the platform (7 cm in diameter) was submerged 1 cm below the water surface. Rats did not swim in the pool before training. Three starting positions were used, and since the 3 day after reperfusion each rat was trained with three trials per day for 7 days. After reaching the platform, the rat was allowed to remain on it for 30 s. If the rat did not find the platform within 90 s, the trial was terminated and the animal was put on the platform for 30 s.

2.5. Western blot analysis

The hippocampus area were homogenized in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). Protein concentration was determined with BCA Protein Assay Kit (Pierce, Rochford, IL). Total proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyphorylated difluoride (PVDF) membrane. The membranes were incubated with 5% bovine serum albumin or skim milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and incubated with a primary antibody diluted in that solution at 4 °C overnight. After being washed with TBST for three times, the membranes were incubated with an HRP-labeled secondary antibody, and developed using the ECL detection Kit (Amersham Biosciences, Piscataway, NJ). The membranes were treated with 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7) at 50 °C for 30 min, and then re-proved with an antibody recognizing different proteins.

Cytosolic and nuclear proteins were prepared by differential centrifugation. Cell pellets resuspended in 5 vol of cytoplasmic extract (CE) buffer [10 mM HEPES (pH 7.5), 60 mM KCl, 1 mM EDTA/1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 0.075% (vol/vol) Igepal CA-630, Sigma] were homogenized by repeated passage through a 23-gauge needle. Homogenate was centrifuged at 500 × *g* for 5 min at 4 °C to pellet out nuclei. Supernatants were centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatants were used as cytoplasmic extracts. Nuclear pellets were washed with 100 μl of CE buffer and resuspended in 1 vol of nuclear extract (NE) buffer [20 mM Tris–HCl, 420 mM NaCl, 60 mM KCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 25% (vol/vol) glycerol; pH 8.0]. Salt concentration was adjusted to 400 mM by addition of 5 M NaCl, followed by addition of 1 vol of NE buffer. Nuclear extracts were incubated for 10 min at 4 °C and centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatants were used as nuclear extracts.

2.6. $[Ca^{2+}]_i$ measurements by multi-photon laser microscopy

Ca^{2+} indicator, Indo-1, was loaded into hippocampal pyramidal cells in the slice using a local ester loading method as described previously (Regehr and Tank, 1991). The main advantage of this method is generally non-invasive and it can