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Time-Window of Progesterone Neuroprotection After Stroke and Its Underlying Molecular Mechanisms

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1. Introduction

Evidence exists for a gender difference in the vulnerability to either stroke or traumatic brain injury (TBI) in humans. For example, pre-menopausal women with the high serum levels of ovarian hormones estrogen (E2) and progesterone (P4) have a lower risk of stroke (Kannel et al., 1994; Sacco et al., 1997) and a better outcome following stroke (Thorvaldsen et al., 1995) or TBI (Groswasser et al., 1998) relative to men of the same age. After menopause, incidence of stroke in women increases abruptly (Wenger et al., 1993) coincident with decreases in the circulating levels of the ovarian steroid hormones, estrogen (E2) and progesterone (P4). Although clinical trial for TBI with P4 treatment has been well tolerated and giving improved outcomes (Wright et al., 2007; Stein et al., 2008), clinical trial with P4 treatment after cerebral stroke has yet to be initiated. There is increasing evidence that P4 exerts a potent neuroprotective effect against ischemia-induced brain injury in experimental models (Chen et al., 1999; Kumon et al., 2000; Morali et al., 2005; Sayeed et al., 2006) when administered either before insult or after the onset of reperfusion (Murphy et al., 2002; Sayeed et al., 2007). Furthermore, the administration of P4 promotes functional recovery after cerebral ischemia (Gibson & Murphy, 2004; Sayeed et al., 2007). Important enough, a single injection of P4 (4 mg/kg) conducted even 2 h after transient focal brain ischemia reduced cortical infarct volumes (Jiang et al., 1996). Our recent study (Cai et al., 2008) has demonstrated that in male rats a single injection of P4 (4 mg/kg) at 1 h or 48 h prior to an experimental stroke shows protective effects against the ischemia-induced neuronal death and the deficits in spatial cognition and LTP induction. However, to date no systematic study has conducted concerning the effects of P4 against brain injury beyond 6 h following the onset of ischemia (Gibson et al., 2008). Therefore, the present study focused on the effective time-window of neuroprotection by P4 treatment, which would give useful information in treating stroke.

Effects of P4 on the brain generally involve three principle mechanisms, including regulation of gene expression, activation of intracellular signal cascades and modulation of

neurotransmitter systems. P4 has been well known to affect transcription processes through the action on the classical nuclear progesterone's receptor (P4R) followed by multiple interactions with DNA and sequence-specific transcription factors (Beato et al., 1995; Guerra-Araiza et al., 2003). The activation of P4R regulates the expression of anti-apoptotic proteins such as bcl-2, and pro-apoptotic genes including bax and bad and caspase-3 (Schlesinger and Saito, 2006). On the transcriptional level, P4 reduces both the nuclear concentration of NF κ B and expression of NF κ B target genes. P4 has been found to influence the activity of many signaling pathways so-called "nongenomic mechanisms" *via* a membrane-associated P4R (mP4R) that lacks functional DNA-binding domain (Guerra-Araiza et al., 2009). Increasing evidence indicates that P4R activates Src-ERK signaling pathway which serves as an indicator of growth factor activity in mammalian breast cancer cells (Boonyaratanakornkit et al., 2008; Faivre and Lange, 2007). Cai et al. (2008) has demonstrated that P4 triggers P4R-mediated long-lasting (> 48 h) phosphorylation of ERK1/2 and enhances the translocation of phosphorelated ERK2 into the nucleus. In addition, rapid effects of P4 is suggested to be mediated by membrane-associated P4-binding protein 25-Dx (Meffre et al., 2005) to increase the level of phosphorylated Akt in neuronal cells (Singh et al., 2001). The membrane-associated P4R component 1 (PGRMC1) has been reported to elevate the level of Akt phosphorylation in breast cancer (Neubauer et al., 2008). P4 increases the phosphorylation of ERK and Akt, and the expression of the regulatory (p85) subunits of phosphoinositide-3 kinase (PI3K) in the brain (Guerra-Araiza et al., 2009). Furthermore, the P4's metabolite allopregnanolone (ALLO) potentiates the GABAergic synapse activity (Ardeshiri et al., 2006). Finally, much attention has recently been attracted to the antagonizing effects of P4 on sigma-1 (σ_1) receptor (Maurice et al., 2006; Monnet & Maurice, 2006).

The objective of the present study was to determine the P4-neuroprotective effect and its effective therapeutic time-window after transient cerebral ischemia. To this end, male animals subjected to 60 min middle cerebral artery occlusion (MCAO) were given a pair of intraperitoneal injections of P4 (4 mg/kg) separated by 8 h starting at 1, 24, 48, 72 or 96 h after the initiation of cerebral ischemia by middle cerebral artery occlusion (MCAO), and the size of brain infarct, loss of pyramidal neurons in the hippocampal CA1 and cognitive performance of the animals were assessed on 7th day after MCAO. Using pharmacologic tools and western blot analysis, molecular mechanisms underlying the P4-neuroprotective effects against ischemia-induced cerebral injury were also investigated.

2. Materials and methods

2.1 Experimental animals

The present studies were approved by Animal Care and Ethical Committee of Nanjing Medical University. All procedures were in accordance with the guidelines of Institute for Laboratory Animal Research of Nanjing Medical University. Male Sprague-Dawley rats (200-250g, Oriental Bio Service Inc., Nanjing, China) before experiments were used throughout the study. We chose to use only adult male rats in the present study to avoid influence of the E2 effects (Nilsen and Brinton, 2003). Animal rooms were maintained on a 12:12 light-dark cycle starting at AM 7:00 and kept at a temperature of 22-23°C. The animals were permitted free access to food and tap water. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Preparation of focal cerebral ischemia model

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). Rats were anesthetized with a mixture of 70% N₂O and 30% O₂ containing 2.5% isoflurane, and were maintained by the inhalation of 1.5% isoflurane during the operation. Briefly, a heat-blunted black monofilament surgical suture (4/0 G) was inserted into the internal carotid artery to occlude the origin of MCA. Adequacy of vascular occlusion and reperfusion was monitored in the front parietal cortex of the occluded side with a multi-channel laser Doppler flow-meter (PF5050 Q4, Perimed, Jarfalla, Sweden). Body and head temperatures were controlled at 37±0.5°C using a water pads. Arterial blood pressure and gases were monitored through a femoral catheter. After 60 min of occlusion, the filament was withdrawn to allow for reperfusion. Sham-operated (sham-op) animals were treated identically, except that MCAs were not occluded.

2.3 Drug administration

P4 was dissolved in dimethylsulfoxide (DMSO), then in sesame oil to a final concentration of 1% DMSO. P4 (4 mg/kg) was intraperitoneally (i.p.) injected. Two injections of P4 with 8 h interval were given starting at 1, 24, 48, 72 or 96 h after the initiation of MCAO (post-MCAO). We selected this low dosage because P4 at this dosage is reported to significantly reduce the ischemic damage and regulate anti-apoptotic gene expression following TBI in rats (Stein, 2008). In addition, our study (Cai et al., 2008) determined that the treatment with the same dosage of P4 increases ERK1/2 phosphorylation.

To analyze the molecular mechanisms underlying the P4-actions, the P4R antagonist RU486 (3 mg/kg) and the 5 α -reductase inhibitor finasteride (20 mg/kg) (Finn et al., 2006) were given by intraperitoneal injection (i.p.) at 30 min before each administration of P4. The MEK inhibitor U0126 (0.5 nmol) and the PI3K inhibitor LY294002 (0.3 nmol) were injected into the cerebroventricle (i.c.v.) at 30 min before each injections of P4. For i.c.v. implantation, rats were anaesthetized with ketamine (80 mg/kg i.p.). A guide cannula (10 mm length, 22 gauge) aiming above the right lateral ventricle was implanted. The inhibitors or vehicle were injected with a stepper-motorized micro-syringe (Stoelting, Wood Dale, IL, USA) at a rate of 0.5 μ l/min. The drugs were prepared freshly on the day of experiment (final volume = 5 μ l/rat). Control rats were given an equal volume of vehicle.

2.4 Histological examination

2.4.1 Infarct volume measuring

Brains were removed on 7th day post-MCAO, sectioned into 5 equidistant slices (2.0-mm-thick), and incubated in a 2% 2,3,5-triphenyle-tetrazoliumchloride (TTC) solution (15 min) to visualize infarcted tissue. Measurements were performed by manually outlining the margins of the infarcted areas. Unstained areas of brain sections were defined as infarcted using the image analysis software NIH-Image 3.12. Briefly, the infarcted area on the ipsilateral side was indirectly measured by subtracting the noninfarcted area in the ipsilateral hemisphere from the total nonischaemic area of the contralateral (nonischaemic) hemisphere. Hemispheric infarcted areas were calculated separately on each coronal slice and scored from 1 to 5, and each such area was defined as a percentage of the affected hemisphere. The infarction volume did not differ significantly across the samples in MCAO-groups ($P > 0.05$).

2.4.2 Pyramidal cells counting

Rats were deeply anesthetized with pentobarbital (50 mg/kg), transcardially perfused with 4% paraformaldehyde at 7th day post-MCAO. The brains were removed, post-fixed for 24 h, and then processed for paraffin embedding. Coronal sections (4- μ m-thick) including the dorsal hippocampus were cut and stained with toluidine blue. Healthy pyramidal cells showing a round cell body with a plainly stained nucleus were counted by eye using a conventional light microscope (PD70) with a 100 \times objective. The number of surviving CA1 pyramidal cells per 1 mm length along the extent of pyramidal layer were counted as neuronal density (cells/mm) (Cai et al., 2008). We also made supplemental examinations on several slices stained with trypan blue that stains dead cells, and obtained essentially the same result as that determined by eye with hematoxylin and eosin (HE) stained slices.

2.5 Behavioral analysis

2.5.1 Rota rod test

The Rota rod test was used to assess the sensorimotor coordination of rodent on 7th day post-MCAO (see Figure 1A) using an accelerating treadmill (TSE Systems, Germany; 3 cm diameter). For Rota rod training sessions, animals were habituated to the Rota rod and trained to remain on the rotating drum (constant speed 6 rpm) for a minimum of 90 s to provide a preoperative baseline. Animals not achieving baseline criteria were excluded from further study. In the testing sessions, animals were placed on the Rota rod, and the rotational speed was set to accelerate from 6 to 19 rpm over 180 s. The latency time to fall (time on rod), namely the time when the animal first fell off the drum, was recorded.

2.5.2 Morris Water Maze (MWM) test

Morris water maze test was performed from 4th day post-MCAO for consecutive 4 days (see Figure 2A) using a swimming pool (diameter: 180 cm; height: 30 cm) filled with water (20°C) to a depth of 15 cm. A transparent plexiglass platform (7 cm in diameter) was submerged with the top located 1 cm below the water surface. Swimming paths were analyzed by a computer system with a video camera (AXIS-90 Target/2; Neuroscience). After reaching the platform, rat was allowed to remain on it for 30 sec. If the rat did not find the platform within 90 sec, the rat was put on the platform for 30 sec. The escape-latency to reach hidden-platform was measured from three trials to provide a single value for each rat.

2.6 Western blot analysis

Rats were decapitated under deep anesthesia with ethyl ether. The hippocampus in ischemic hemisphere was taken quickly, then homogenized in a lysis buffer containing 50 mM TrisHCl (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, USA). Total proteins (20 μ g) 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 5% nonfat dried milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 60 min at room temperature, and then were incubated with a mouse monoclonal anti-phospho-ERK1/2 antibody (diluted 1:2500, Cell Signaling, Beverly, MA) 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). Following visualization, the blots were stripped by incubation in stripping buffer (Restore, Pierce Chemical Co, Rockford IL) for 5 min, re-blocked for 60 min with 5% nonfat dried milk at room temperature, then incubated with anti-total ERK1/2 (diluted 1:5000, Cell Signaling, Beverly, MA). In each experiment, levels of both ERK1/2 and phosphorelated ERK1/2 (phospho-ERK1/2) were measured in the hippocampus of ischemic hemisphere in MCAO-rats and sham-op rats (control). For each animal, phospho-ERK1/2 was normalized by respective ERK1/2 protein. Each experimental group contained 12 rats. The Western blot bands were scanned and analyzed with the image analysis software package, NIH Image.

2.7 Data analysis/statistics

Data were retrieved and processed with the software Microcal Origin 6.1. The group data are expressed as the means \pm standard error (SE). For comparison between two groups the 2-sided student t-test was used. For comparison between more than 2 groups one-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test was performed. Statistical analysis was performed using the software State7 (STATA Corporation, USA). For the analysis of Morris water maze test, statistical differences were determined by an ANOVA with repeated measures, followed by the Bonferroni post hoc test. Statistical analysis was performed using the State7 software (Stata Corporation, USA). Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1 Effective time-window of P4 against ischemic brain infarct and motor dysfunction

To examine the effects of P4 on ischemia-induced brain infarction, a pair of injections (i.p.) of P4 (4 mg/kg) with an 8 h interval were given starting at 1, 24, 48, 72 or 96 h post-MCAO (Figure 1A). On 7th day post-MCAO the results of TTC staining showed that the 60 min MCAO caused approximately 34% brain infarction mainly in the striatum and the frontoparietal cortex (Figure 1B). In comparison with vehicle-treated MCAO-rats, infarct volumes were significantly decreased by the administration of P4 at 1 and 24 h ($P < 0.01$, $n = 12$) or 48 and 72 h post-MCAO ($P < 0.05$, $n = 12$), but not at 96 h ($P > 0.05$, $n = 12$). Similarly, the performance of rota rod test on 7th day post-MCAO perfectly restored in MCAO-rats treated with P4 at 1, 24, 48 ($P < 0.01$, $n = 12$) and 72 h post-MCAO ($P < 0.05$, $n = 12$; Figure 1C) compared to vehicle-treated MCAO-rats. By contrast, P4 when administered at 96 h post-MCAO had no effect on the ischemia-induced motor impairment ($P > 0.05$, $n = 12$). The results indicate that the administration of P4 after stroke exerts a powerful neuroprotection against ischemia-induced brain damages with a wide effective time-window up to 72 h.

3.2 Effective time-window of P4 against ischemic death of neuronal cells and cognitive impairment

Consistent with the previous report (Cai et al., 2008), the number of hippocampal CA1 pyramidal neurons in ischemic hemisphere decreased to approximately 50% of sham-op hemisphere on 7th day post-MCAO ($P < 0.01$, $n = 8$; Figure 2B). To examine the effects of P4 on ischemia-induced death of pyramidal neurons and impairment of spatial memory, a pair of injections (i.p.) of P4 (4 mg/kg) with an 8 h interval was given at 1, 24, 48, 72 or 96 h

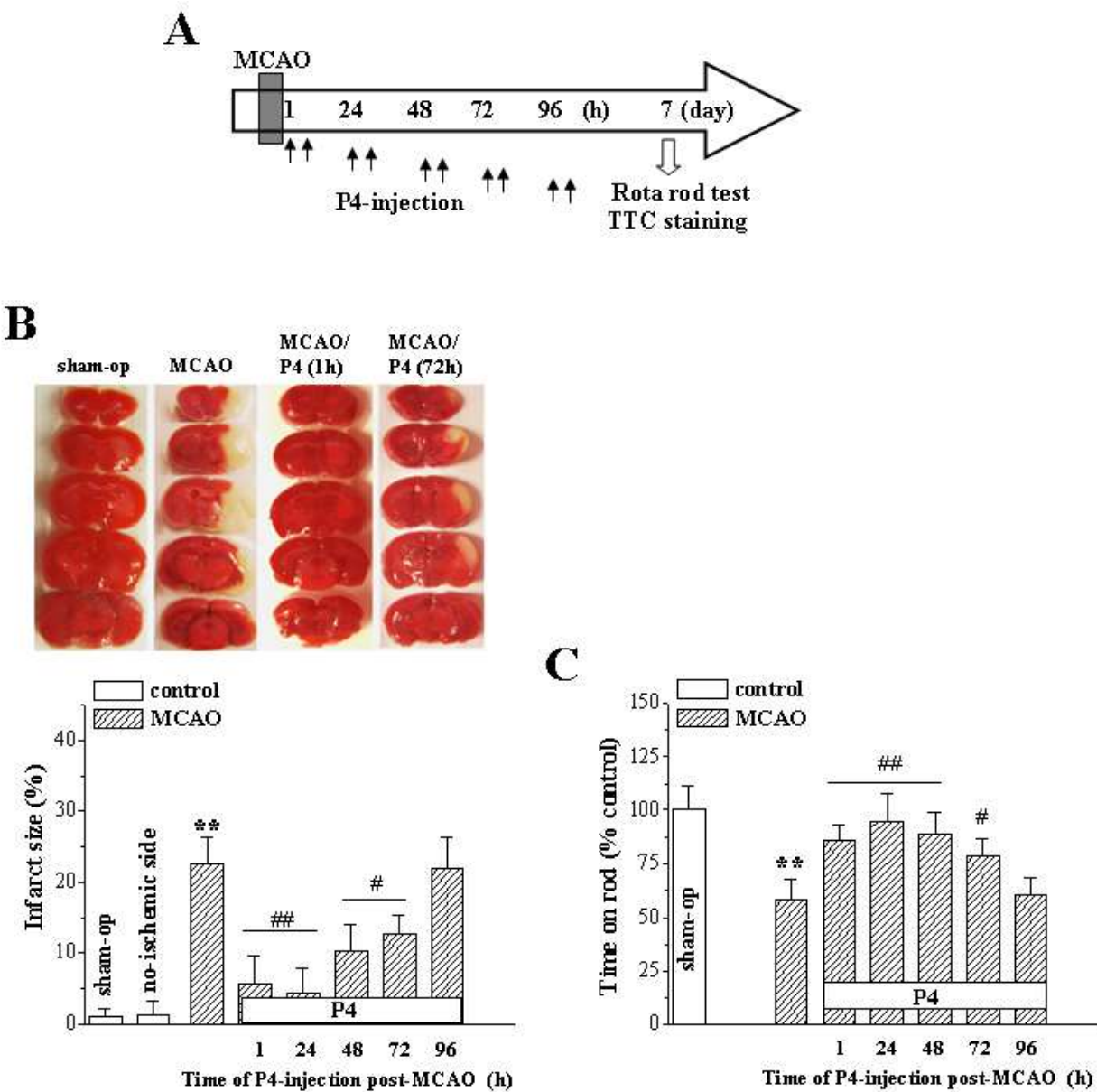


Fig. 1. Effects of P4 on ischemia-induced brain infarct and motor dysfunction. (A) Time chart of experimental procedure in Figure 1B&C. Two injections (i.p.) of P4 (4 mg/kg) with 8 h interval (black arrows) were given starting at 1, 24, 48, 72 or 96 h post-MCAO. (B) Time-window of P4-effect against MCAO-induced brain infarct. Representative pictures of TTC-staining in sham-op rats, MCAO-rats, P4-treated MCAO-rats (upper panels). Bar graph shows the size of brain infarct that was expressed as percentage of the non-infarcted hemisphere on 7th day post-MCAO. Horizontal hollow bar: P4 administration. (C) Time-window of P4-effect against ischemic motor dysfunction. Bar graph shows time on rod in sham-op (open bar) and MCAO-rats (hatched bars) on 7th day post-MCAO. ** $P<0.01$ vs. sham-op rats; # $P<0.05$ and ## $P<0.01$ vs. MCAO-rats.

post-MCAO. The number of dead pyramidal cells was significantly reduced by the treatment with P4 at 1, 24 and 48 h ($P<0.01$, $n=8$) or 72 h post-MCAO ($P<0.05$, $n=8$) compared to vehicle-treated MCAO-rats. However, the administration of P4 at 96 h post-MCAO exerted no significant effect in reducing the number of ischemia-induced loss of

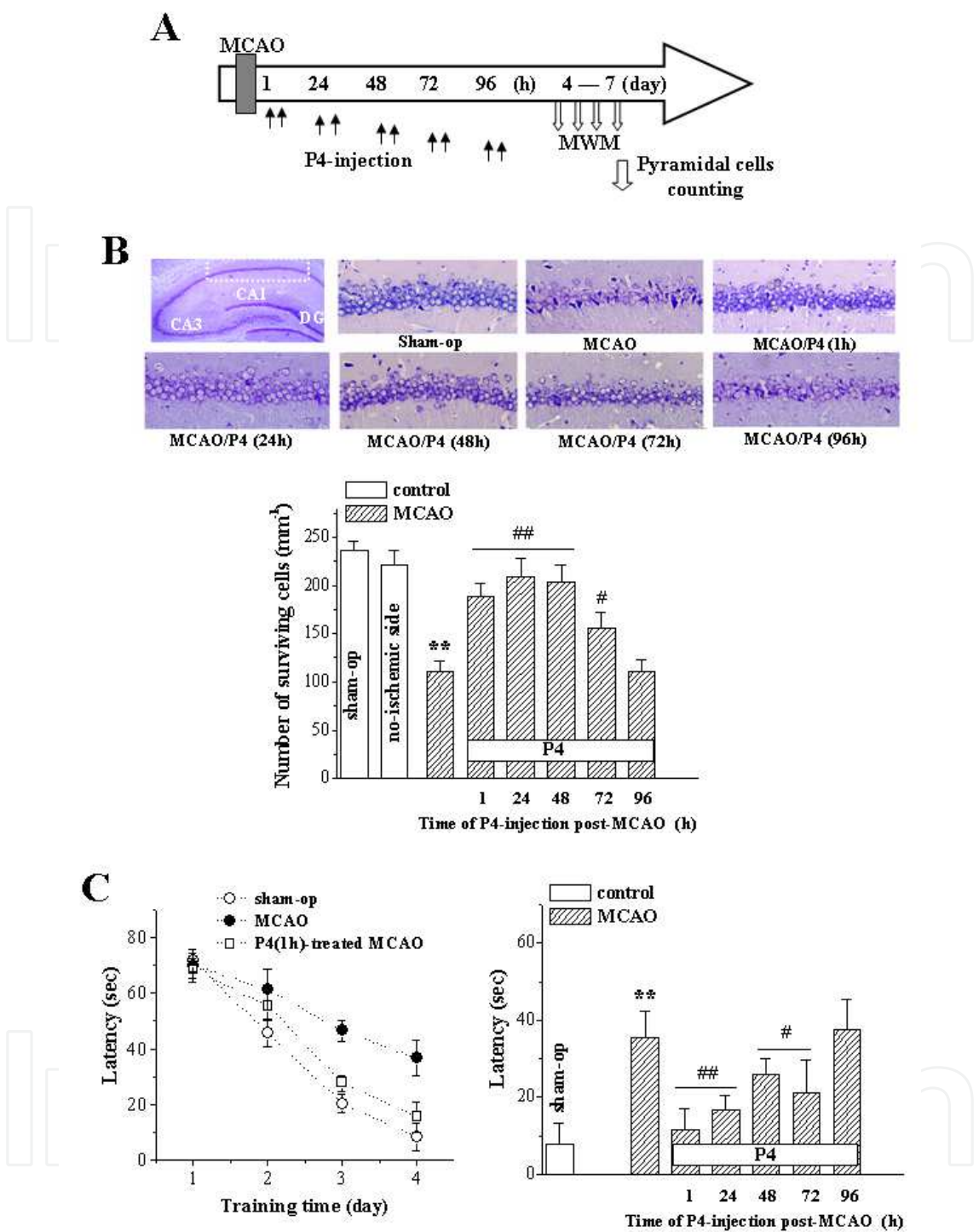


Fig. 2. Effects of P4 on ischemia-induced neuronal cell death and cognitive impairment. (A) Time chart of experimental procedure in Figure 2B&C. Two injections (i.p.) of P4 (4 mg/kg) with 8 h interval (black arrows) were given starting at 1, 24, 48, 72 or 96 h post-MCAO. (B) Time-window of P4-effect against MCAO-induced neuronal cell death. Representative pictures of hippocampal CA1 region in sham-op rats, MCAO-rats, P4-treated MCAO-rats (upper panels). Scale bar=100µm. Bar graph shows density of surviving neurons in the hippocampal CA1 on 7th day post-MCAO. Horizontal hollow bar: P4 administration. (C) Time-window of P4-effect against MCAO-induced deficits in spatial memory. Typical

trials of “Morris” water maze test (left panel) show latency (sec) to reach the hidden-platform against training time (day, day 4-7 post-MCAO) in sham-op rats, MCAO-rats, P4 (1h post-MCAO)-treated MCAO-rats. Bar graph shows the mean latency (\pm SEM) to reach the hidden-platform on 4th day post-training. ** $P < 0.01$ vs. sham-op rats; # $P < 0.05$ and ## $P < 0.01$ vs. MCAO-rats.

pyramidal cells ($P > 0.05$, $n = 8$). The P4 administration per se caused no observable change in CA1 pyramidal neurons on 7th day after sham-op. Spatial learning and memory function was examined by the Morris water maze test from 4th day post-MCAO for consecutive 4 days (Figure 2A). In comparison with sham-op rats, the escape-latency to reach the hidden-platform on 7th day post-MCAO increased approximately 2-fold ($P < 0.01$, $n = 8$; Figure 2C). The behavior of acquisition performance coincided with the histological changes; the prolongation of escape-latency was perfectly improved by the treatment with P4 at 1, 24 and 48 h post-MCAO ($P < 0.01$, $n = 8$), while was partially reduced by the injection of P4 at 72 h post-MCAO ($P < 0.05$, $n = 8$). By contrast, the administration of P4 at 96 h post-MCAO failed to affect the prolonged escape-latency ($P > 0.05$, $n = 8$). Both the histological and behavioral examinations here strongly suggest that the effective time-window of the neuroprotection by the P4 treatment is spanning from 1 h to 72 h post-MCAO. In the following sections, we describe the results on the analyses of the molecular mechanisms underlying the P4 affording neuroprotective effects on ischemia-induced death of pyramidal cells.

3.3 P4-neuroprotection at 1 h post-MCAO is mediated by its metabolite ALLO

A recent study (Ciriza et al., 2006) has revealed that the neuroprotection by P4 after ischemic brain injury is abolished by finasteride, a 5 α -reductase inhibitor that inhibits the conversion of P4 to allopregnanolone (ALLO). To determine whether P4 exerts neuroprotection through its metabolite ALLO, finasteride (20 mg/kg i.p.) was given at 30 min prior to every P4-injection. The results showed that the pre-treatment with finasteride partially attenuated the neuroprotection of P4 at 1 h post-MCAO against MCAO-induced neuronal death ($P < 0.05$, $n = 8$; Figure 3A) and prolongation of escape-latency ($P < 0.05$, $n = 8$; Figure 3B), but it did not

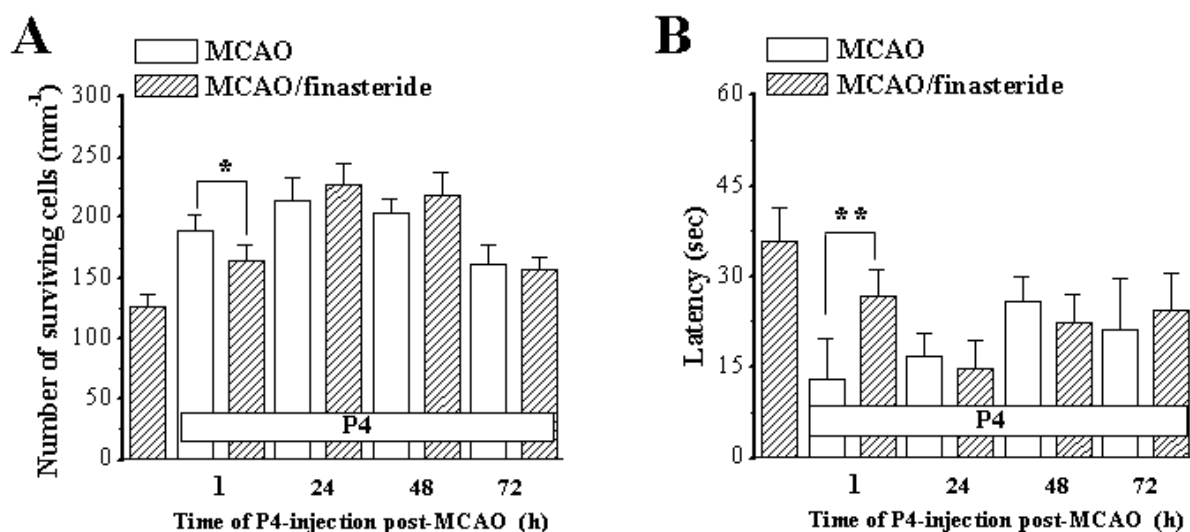


Fig. 3. Effects of finasteride, a 5 α -reductase inhibitor, on the neuroprotection of P4 against MCAO-induced neuronal cell death (A) and cognitive impairment (B). Horizontal hollow bar: P4 administration. Animals were treated with finasteride at 30 min before every P4-injection. Note that the P4-neuroprotection at 1 h post-MCAO is partially blocked by

finasteride. * $P < 0.05$ and * $P < 0.01$ vs. P4-treated MCAO-rats at 1 h post-MCAO. affect the neuroprotection by P4 at 24, 48 or 72 h post-MCAO ($P > 0.05$, $n = 8$). The results indicate that the neuroprotection by P4 at 1 h post-MCAO is, if not all, caused by a protective action of its metabolite ALLO against ischemia-induced brain damage.

3.4 P4-neuroprotection at 24 and 48 h post-MCAO is mediated by P4R activation

The neuroprotection by P4 administered at 48 h pre-MCAO has been known to depend on P4R function (Faivre and Lange, 2007). To test this possibility in our case, the nuclear P4R blocker RU486 (3 mg/kg, i.p.) was given at 30 min prior to each P4 injection. The results showed that the pre-treatment with RU486 abolished the neuroprotective effects of P4 at 24 and 48 h post-MCAO against ischemia-induced neuronal death ($P < 0.01$, $n = 8$; Figure 4A) and spatial memory impairment ($P < 0.01$, $n = 8$; Figure 4B), whereas it failed to affect the neuroprotection by P4 at 1 or 72 h post-MCAO ($P > 0.05$, $n = 8$). Meanwhile, in the absence of P4 the administration of RU486 at 24 or 48 h post-MCAO had no effect on either neuronal death ($P > 0.05$, $n = 8$) or spatial cognitive function ($P > 0.05$, $n = 8$). These results suggest that the neuroprotective effect of P4 administered at 24 and 48 h post-MCAO involves the P4R-mediated mechanism.

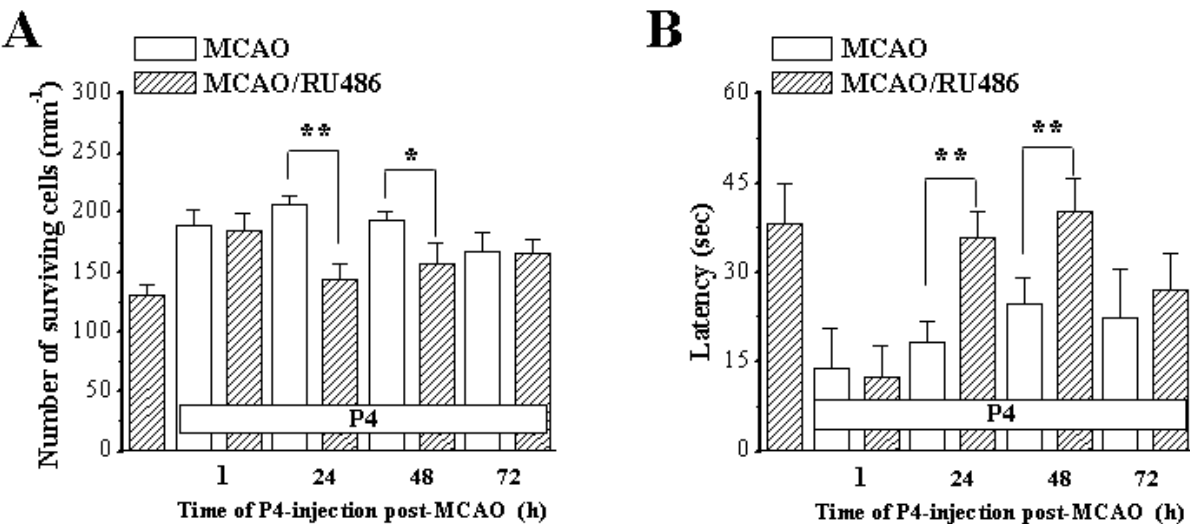


Fig. 4. Effects of RU486, a R4R antagonist, on the neuroprotection of P4 against MCAO-induced neuronal cell death (A) and cognitive impairment (B). Horizontal hollow bar: P4 administration. Animals were treated with RU486 at 30 min before every time P4-injection. Note that the P4-neuroprotection at 24 and 48 h post-MCAO is blocked by RU486. * $P < 0.05$ and * $P < 0.01$ vs. P4-treated MCAO-rats at 24 and 48 h post-MCAO.

3.5 P4-neuroprotection at 24 and 48 h post-MCAO depends on P4R-ERK signaling

As P4R-mediated ERK1/2 activation protects the ischemic brain damage (Cai et al., 2008), the experiment was designed to explore the involvement of ERK1/2 in the P4R-dependent neuroprotection after MCAO. Expectedly, the ERK kinase (MEK) inhibitor U0126 (0.3 nmol, i.c.v.) blocked the neuroprotection by P4 at 24 and 48 h against MCAO-induced neuronal death ($P < 0.01$, $n = 8$; Figure 5A) and spatial memory impairment (P4 at 24 h post-MCAO, $P < 0.01$, $n = 8$; P4 at 48 h post-MCAO, $P < 0.05$, $n = 8$; Figure 5B), whereas it failed to affect the

P4R-independent neuroprotection exerted by P4 administered at 1 or 72 h post-MCAO ($P>0.05$, $n=8$). These results indicate that the P4R-mediated neuroprotection is highly coupled with ERK1/2 signaling pathway.

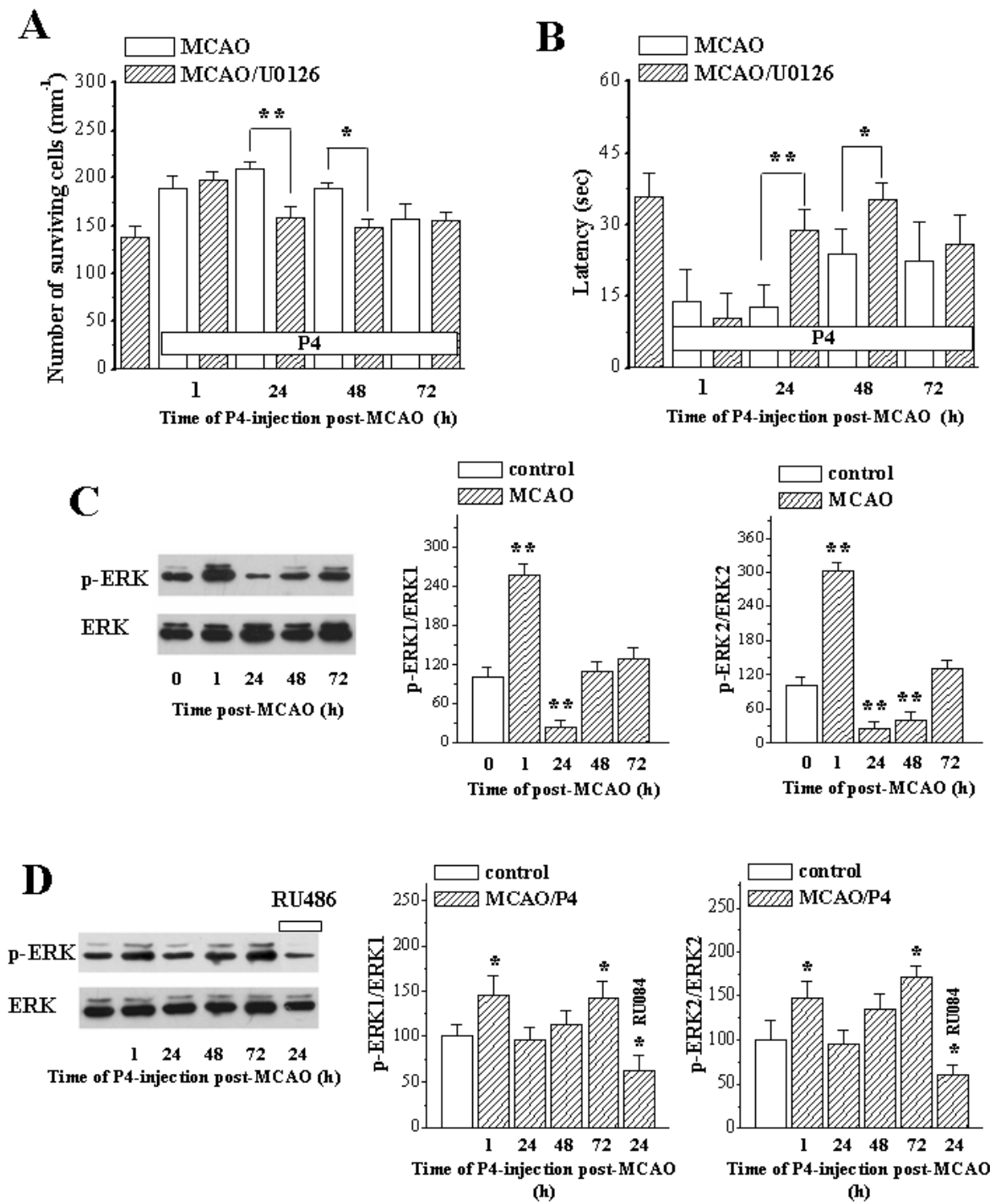


Fig. 5. Effects of U0126, a MEK inhibitor, on the neuroprotection of P4 against MCAO-induced neuronal cell death (A) and cognitive impairment (B). Horizontal hollow bar: P4 administration. Animals were treated with U0126 at 30 min before every time P4-injection. Note that the phospho-ERK1/2 at 24 and 48 h post-MCAO is blocked by U0126. * $P<0.05$ and ** $P<0.01$ vs. P4-treated MCAO-rats at 24 and 48 h post-MCAO. (C) Kinetics of hippocampal

phospho-ERK1/2 at 1, 24, 48 and 72 h post-MCAO. Representative western blots represent ERK1/2 phosphorylation immunoreactivity obtained from whole-cell lysates. Level of phospho-ERK1/2 is expressed as a percentage of phospho-ERK1/2 in sham-op rats. (D) Effect of P4 on phospho-ERK1/2 at 1, 24, 48 and 72 h post-MCAO. P4 was given at 30 min before harvested hippocampus. Values of phospho-ERK1/2 were normalized by phospho-ERK1/2 in sham-op rats. * $P < 0.05$ and ** $P < 0.01$ vs. sham-op group; # < 0.05 vs. MCAO-rats treated with P4 at 24 h post-MCAO. Horizontal axis: Time of post-MCAO p4 injection.

Emerging evidence indicates that transient cerebral ischemia promotes the dephosphorylation of ERK1/2 (Jover-Mengual et al., 2007). To confirm this, kinetics of hippocampal ERK1/2 phosphorylation (phospho-ERK1/2) after MCAO was measured using Western blot analysis. In comparison with that before MCAO, the level of phospho-ERK1/2 was largely increased at 1 h post-MCAO ($P < 0.05$, $n = 12$; Figure 5C), followed by a persistent decrease at 24 h and 48 h post-MCAO ($P < 0.05$, $n = 12$), then returned to the basal level at 72 h post-MCAO ($P > 0.05$, $n = 12$). To investigate the effects of P4 on the changes in ERK1/2 phosphorylation after MCAO, the MCAO-rats were given a single injection of P4 at 1, 24, 48 or 72 h post-MCAO. Thirty minutes after the P4-injection hippocampal preparations were harvested to measure phospho-ERK1/2. As shown in Figure 5D, the treatment with P4 slightly attenuated the increased phospho-ERK1/2 at 1 h post-MCAO ($P < 0.05$, $n = 12$), perfectly rescued the reduction of phospho-ERK1/2 at 24 and 48 h post-MCAO ($P < 0.01$, $n = 12$), and elevated the level of phospho-ERK1/2 at 72 h post-MCAO ($P < 0.05$, $n = 12$). The P4R antagonist RU486 could block the protective effect of P4 on the reduction of phospho-ERK1/2 at 24 h post-MCAO ($P < 0.05$ vs. P4-treated MCAO-rats, $n = 12$). These observations clearly indicate that the P4R-dependent neuroprotection against ischemia-induced brain damage is mediated, at least in part, through the regulation of ERK1/2 activity.

3.6 P4-neuroprotection at 24–72 h post-MCAO requires PI3K signaling

As P4 increases the level of Akt-phosphorylation, a partial process of PI3K signaling (Guerra-Araiza et al., 2009), the specific PI3K inhibitor LY294002 (0.3 nmol) was injected into the cerebri-ventricle (i.c.v.) at 30 min prior to P4 injection to examine the involvement of PI3K-Akt signaling pathway in the P4-neuroprotection. The results showed that the pre-treatment with LY294002 partially attenuated the neuroprotection of P4 at 24 and 48 h post-MCAO ($P < 0.05$, $n = 8$; Figure 6A) and completely abolished the neuroprotection of P4 at 72 h post-MCAO ($P < 0.01$, $n = 8$), whereas it had no effect on the neuroprotection of P4 at 1 h post-MCAO ($P > 0.05$, $n = 8$). Furthermore, the pre-treatment with LY294002 blocked the P4-improved impairment of spatial memory when administered at 24, 48 and 72 h post-MCAO ($P < 0.05$, $n = 8$; Figure 6B). The results indicate that the PI3K-Akt signaling is involved in the P4R-dependent and P4R-independent neuroprotections by P4, depending on the timing of P4 injection.

4. Discussion

The present study provides evidence that the treatment with P4 after transient brain ischemia exerts a powerful neuroprotection with a wide effective time-window up to 72 h post-MCAO. The neuroprotective effects of P4 were mediated by different molecular mechanisms depending on the timing of P4 administration after ischemia. The neuroprotection by P4 at 1 h post-MCAO appeared to be caused through P4's metabolite

allopregnanolone (ALLO) because the protection was significantly attenuated by the 5 α -reductase inhibitor finasteride. The neuroprotection of P4 at 24 and 48 h post-MCAO appeared to be P4R-dependent through rescuing the down-regulation of ERK1/2 phosphorylation after stroke. The neuroprotective effects of P4 at 72 h post-MCAO required PI3K activation in a P4R-independent way.

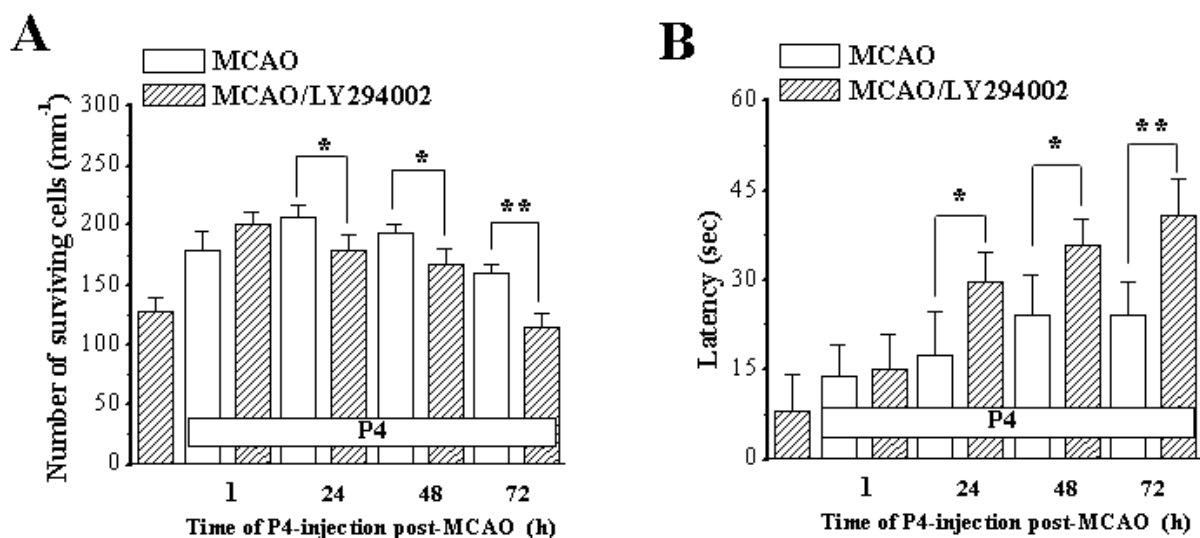


Fig. 6. Involvement of PI3K in P4-neuroprotection. Animals were treated with the specific PI3K inhibitor LY294002 (LY) at 30 min before administration of P4. Horizontal hollow bar: P4 administration. Note that the P4-neuroprotection at 24–72 hr post-MCAO requires PI3K signaling. * $P < 0.05$ and ** $P < 0.01$ vs. MCAO-rats treated with P4 at 24, 48 and 72 hr post-MCAO.

4.1 Anti-excitotoxic effect of P4's metabolite ALLO at 1 h after MCAO

One recent report indicates that either P4 or ALLO when administered at 2 h post-MCAO is effective in reducing the infarct volume after focal brain ischemia, where ALLO shows more effective neuroprotection than its parent compound (Sayeed et al., 2007). Similarly, our results in the present study showed that the neuroprotection of P4 at 1 h post-MCAO was sensitive to the 5 α -reductase inhibitor finasteride. Thus, it is proposed that the acute neuroprotection of P4 within 1–2 h ischemia/reperfusion is caused by ALLO, a positive regulator of GABAA receptor (Belelli & Lambert, 2005). This notion is supported by an earlier study (Ardeshiri et al., 2006) showing that the GABAA receptor antagonist picrotoxin could prevent the neuroprotection afforded by P4. The P4 neuroprotection mainly focuses on some populations of neurons that are sensitive to excitotoxicity, including the pyramidal neurons in the hippocampus and cerebral cortex, Purkinje cells in the cerebellum, as well as the neurons in the dorsal striatum and the caudate nucleus (Monnet and Maurice, 2006; Schumacher et al., 2007). Immediately after ischemia, excessive presynaptic glutamate releases result in the accumulation of extracellular glutamate to reach concentrations that induce over-activation of glutamate receptors called excitotoxicity (Jabaudon et al., 2000; Phillis and O'Regan, 2003). The process of excitotoxicity has been demonstrated in several experimental models of cerebral ischemia (Butcher et al., 1990). Therefore, it is highly likely that P4 and ALLO at 1 h post-MCAO prevent the brain injury by suppressing over-excitation of pyramidal neurons through the activation of GABA_A receptors.

However, we noted that the treatment with finasteride could not completely block the P4-neuroprotection at 1 h post-MCAO (see Figure 3A). Excessive presynaptic glutamate releases after cerebral ischemia lead to neuronal death mainly by excessive calcium entry through N-methyl-D-aspartate receptor (NMDAr). Our recent study (Cai et al., 2008) has revealed that P4, as a potential σ_1 receptor antagonist (Monnet and Maurice, 2006), reduces Ca^{2+} influx across NMDAr-channels to protect hippocampal neurons from ischemia-induced cell death. In addition, at 1 h after brain ischemia the activation of σ_1 receptor by PRE-084, a σ_1 receptor agonist, exacerbates ischemia-induced neuronal cell death in an NMDAr-dependent manner (Li et al., 2009). However, conflicting results have reported that the activation of σ_1 receptor enhances presynaptic glutamate release in the hippocampal CA1 (Meyer et al., 2002), and promotes the Ca^{2+} influx across NMDAr-channels (Monnet et al., 2003) and the Ca^{2+} efflux from calcium pools via inositol 1,4,5-trisphosphate receptors (Su and Hayashi, 2003). This discrepancy may be due to the difference in experimental condition or timing of P4 action. Further studies are required to directly observe the P4 effects on NMDAr- Ca^{2+} influx in an acute phase after stroke.

4.2 P4R-dependent ERK activation at 24–48 h after stroke

Our results revealed that the P4R-mediated ERK1/2 signaling was involved in the neuroprotection by P4 at 24–48 h post-MCAO. P4R ligand has been demonstrated to induce a transient (5–10 min after P4-application) activation of Src-Ras-ERK1/2 and a persistent (6–72 h) ERK1/2 activation (Faivre and Lange, 2007). Recently, Cai et al. (2008) provided in vivo evidence that P4 acts on P4R to trigger a long-lasting (> 48 hr) phosphorylation of ERK1/2, resulting in a promoted translocation of phosphorelated ERK2 into the nucleus. The translocation of ERK1/2 is a pivotal and necessary process for the activation of cAMP response element binding protein (CREB) (Nilsen and Brinton, 2003). The ERK1/2-CREB signaling has been implicated to play a critical role in the brain ischemic tolerance (Gonzalez-Zulueta et al., 2000) and neuronal cell survival (Singh, 2005; 2006). The CREB cascade can increase the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-XL (Yao et al., 2005) and decrease the expression of pro-apoptotic molecules such as Bax, Bad and caspase-3 (Djebaili et al., 2004). Consistent with the results reported by Jover-Mengual et al. (2007), we in the present study showed a decreased activity of ERK1/2 at 24 and 48 h post-MCAO. More importantly, the activation of P4R at 24–48 h after ischemia could rescue the down-regulation of ERK1/2. Therefore, it is highly likely that the P4R-dependent neuroprotection is closely coupled with ERK1/2 signaling.

On the other hand, western blot analysis showed a transient elevation of ERK1/2 phosphorylation at 1 h post-MCAO. The elevation of ERK1/2 activity immediately after stroke has also been observed in humans (Slevin et al., 2000) and a rat model of cerebral ischemia (Wang et al., 2003), in which increased intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) after ischemia seem to lead hyper-activation of ERK1/2. Alessandrini et al. (1999) and Namura et al. (2001) provided evidence for a neuroprotective role of MEK inhibitor following transient ischemia as manifested by the reduction of infarct size and improvement of functional outcome. The neuroprotection by MEK-inhibition in ischemic brain is associated with an activation of potential anti-apoptotic pathway that suppresses caspase-3 activation and apoptosis (Wang et al., 2003). To our surprise, we observed that the treatment with P4 could attenuate the elevation of ERK1/2 phosphorylation at 1 h post-MCAO. Because the neuroprotection by P4 at 1 h post-MCAO was P4R-independent, it is proposed that P4

prevents ischemia-increased $[Ca^{2+}]_i$ by antagonizing σ_1 receptor, which may stabilize the ERK1/2 activation.

4.3 PI3K signaling is required for P4R-independent and P4R-dependent neuroprotections

P4 has been reported to enhance the phosphorylation of Akt/PKB (Singh et al., 2001; Kuolen et al., 2008) in the hippocampus and cerebellum (Guerra-Araiza et al., 2009). On the other hand, PI3K signaling is believed to suppress apoptotic cell death via its downstream effectors, such as Akt/PKB, to inhibit the Bcl2 family protein Bad (Noshita et al., 2001). Using the specific PI3K inhibitor LY294002, the present study provided in vivo evidence that the PI3K signaling is required for the P4-neuroprotection at 24–72 h post-MCAO against ischemia-induced brain damage. In human breast cancer cells, P4 induces rapid and transient activation of PI3K-Akt pathway in a P4R-dependent manner (Migliaccio et al., 1998; Castoria et al., 2001). It was reported that progestins rapidly activated PI3K-Akt pathway via P4R (Vallejo et al., 2005; Ballare et al., 2006). However, our results here showed that the PI3K-mediated neuroprotection by P4 administered at 72 h post-MCAO is P4R-independent. P4 is reportedly to regulate PI3K signaling pathway through its metabolites (Guerra-Araiza et al., 2009), but our data determined that the neuroprotection of P4 at 24–72 h post-MCAO was insensitive to the inhibition of 5α -reductase by finasteride. P4-binding membrane protein 25-Dx (also known as PGRMC1) in the brain (Krebs et al., 2000; Sakamoto et al., 2004) is involved in the anti-apoptotic actions of P4 (Peluso et al., 2006, 2008). Further studies are needed to elucidate whether P4 cascades PI3K signaling after stroke through P4-binding 25-Dx mechanisms.

4.4 Clinical significance

P4 treatment after ischemia at relatively a low dose (4 mg/kg) exerts powerful neuroprotective effects with a wide, at least up to 72 h post ischemia, effective time-window, which would provide a great benefit in treating stroke. The present study provides evidence that the P4 neuroprotection has a wide effective time-window that is realized by a time dependent multiple neuroprotective mechanisms after ischemia. The results shown here not only help to understand the correlation between the declined level of P4 and the abruptly increasing incidence of stroke following the menopause, but also provide a novel therapeutic opportunity of P4 against the ischemic brain injury.

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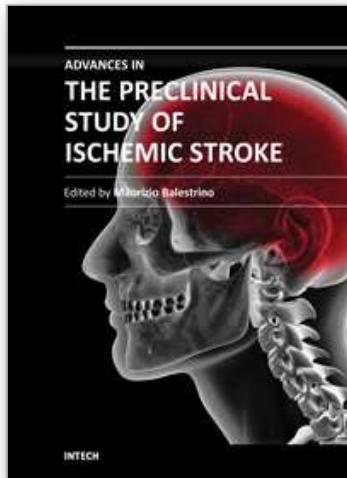
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