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DELAYED ADMINISTRATION OF A PTEN INHIBITOR BPV IMPROVES FUNCTIONAL RECOVERY AFTER EXPERIMENTAL STROKE

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Abstract—Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) inhibitors administered prior to or immediately after experimental stroke confer acute neuroprotection. However, it remains unclear if delayed treatment with a PTEN inhibitor improves long-term functional recovery after stroke. We addressed the issue in this study. Adult male mice were subjected to 1 h of middle cerebral artery occlusion (MCAO) followed by treatment with a well-established PTEN inhibitor BPV or saline daily for 14 days, starting at 24 h after MCAO. Functional recovery was assessed with behavioral tests and acute infarct volumes were analyzed histologically. Delayed BPV treatment did not reduce infarction during the acute phase, but significantly improved long-term functional recovery after MCAO. Since PTEN is a critical intrinsic inhibitory factor in axonal regeneration, we further examined BPV effects on axonal densities following MCAO using bielschowsky silver staining and immunohistochemistry with antibodies against myelin basic protein. Delayed BPV treatment significantly increased axon densities in the ischemic brain at 14 days after MCAO. Moreover, PTEN expression persistently remained high in the ischemic brain over 14 days after MCAO, and BPV treatment increased post-ischemic activation of Akt and mTOR in the ischemic brain. Akt and mTOR activation are the well-established cascades downstream to PTEN inhibition and have been shown to contribute to post-injury axonal regrowth in response to PTEN inhibition. Consistently, in an *in vitro* neuronal ischemia model, BPV enhanced axonal outgrowth of primary cortical neurons after oxygen-glucose deprivation and the enhancing effects were abolished by Akt/mTOR inhibition. In conclusion, delayed BPV treatment improved functional recovery from experimental stroke possibly via enhancing axonal growth and Akt/mTOR activation contributed to BPV-enhanced post-stroke axon growth.

Key words: PTEN inhibitor, BPV, functional recovery, axonal densities, stroke.

INTRODUCTION

Stroke is a major cause of death and disabilities globally, yet therapeutic approaches are rather limited. For instance, the only approved drug for ischemic stroke is tissue plasminogen activator, which must be administered within 4.5 h of stroke onset. Thus, there is an unmet need for stroke therapies that could be commenced beyond this therapeutic window (Moskowitz et al., 2010). Several strategies have been suggested for extending the therapeutic windows of stroke therapies. The one that is drawing growing interest is to develop the therapies that aim at enhancing post-stroke functional recovery.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual specificity phosphatase that dephosphorylates both lipids and phosphoproteins. By activating Akt (Li et al., 2009) or preserving γ-aminobutyric acid subtype A receptors (Liu et al., 2010a), PTEN inhibitors administered prior to or immediately after experimental stroke confer acute neuroprotection following cerebral ischemia. Interestingly, PTEN may also serve as a restorative target since emerging data show that PTEN deletion induces axonal regrowth following both CNS and peripheral nerve injuries (Park et al., 2008; Christie et al., 2010; Liu et al., 2010b). However, it is currently not clear whether PTEN inhibitors improve long-term functional recovery after stroke, nor is it clear whether the therapeutic window of PTEN inhibitors could be beyond 4.5 h following ischemic stroke.

Thus, we investigated if delayed treatment with a well-established PTEN inhibitor BPV (Schmid et al., 2004; Li et al., 2009; Christie et al., 2010; Liu et al., 2010a) improves long-term functional recovery following

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Abbreviations: IBZ, ischemic boundary zone; IP, intraperitoneal; MBP, myelin basic protein; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score; OGD, oxygen-glucose deprivation; PCN, primary cortical neuron; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

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cerebral ischemia. To explore the possible mechanisms underlying BPV restorative effects, we also investigated if delayed BPV treatment increases post-ischemic axonal densities in the ischemic boundary zone (IBZ) where neural repair is thought to occur following cerebral ischemia.

EXPERIMENTAL PROCEDURES

Transient middle cerebral artery occlusion (MCAO) and drug administration

All animal experiments were approved by Ethical Review Panels of Changhai Hospital and Soochow University and subjected to the Experimental Animal Act, 1988. To determine the restorative effects of BPV, adult male CD-1 mice weighing 30 ± 2 g received 1 h intraluminal MCAO according to previous publications (Chen et al., 2001b; Gibson and Murphy, 2004; Ren et al., 2011). In brief, mice were anesthetized and body temperature was maintained by warming pads. A lysine-coated nylon monofilament with a heat-blunted tip (diameter 0.22 ± 0.02 mm) was inserted into the right internal carotid artery via the external carotid artery. The filament was secured and the surgical site was closed when the tip of the filament reached the origin of the middle cerebral artery. After 60 min of occlusion, the filament was withdrawn to allow for reperfusion. Vascular occlusion (< 30% of baseline) and reperfusion (> 75% of baseline) were verified with laser Doppler flowmetry (PeriFlux System 5000, Perimed Inc., Stockholm, Sweden) by affixing a laser probe to the mouse skull to monitor cortical perfusion. Sham-operated mice received identical surgery with the exception of filament insertion to produce occlusion.

At 24 h after reperfusion, neurological deficits were assessed using modified neurological severity score (mNSS). Mice showing neurological deficits were randomly divided into two groups to receive: (1) intraperitoneal (IP) injection of the PTEN inhibitor [BPV (phen)] (EMD Chemicals Inc., Gibbstown, NJ, United States) at a dose of 0.2 mg/kg/day for 14 days, starting at 24 h after MCAO; or (2) an equal volume of saline. IP injection of BPV at this concentration has been shown to inhibit cerebral PTEN and confer neuroprotection following experimental stroke (Li et al., 2009; Shi et al., 2011). Over 14 days after MCAO, the mortalities of BPV- and saline-treated groups were: 12 out of 42 and 22 out of 42 mice, respectively. Gross examination revealed that, regardless of treatments, most mice died from lung infection after MCAO.

Behavioral testing

mNSS were examined in BPV-treated mice (n = 12) and saline-treated mice (n = 12) before and at 1, 3, 5, 7, 9, 11 and 14 days after MCAO in a blinded manner. mNSS is a comprehensive test for evaluating motor, sensory, reflex and balance abilities. Neurological deficits were graded on a scale of 0 to 18. Table 1 describes the set of mNSS in detail (Chen et al., 2001a; Zhang et al., 2010). According to Table 1, score points were awarded when mice were unable to perform the tests or lacked tested reflexes. Thus, the higher the scores are, the more severe the injury is.

Limb placing, a test initially used for assessing lateralized sensorimotor dysfunction of rats after experimental stroke, has been translated to the mouse MCAO model recently (Jin et al., 2010). We performed the forelimb placement test in BPV-treated mice (n = 12) and saline-treated mice (n = 12) before and at 1, 3, 5, 7, 9, 11 and 14 days after MCAO, as previously described for MCAO-treated mice (Wang et al., 2009; Jin et al., 2010). Briefly, mice were brought laterally toward the benchtop, allowing for spontaneous placement of the forelimbs. Mice were then gently pulled down, forcing the limbs away from the bench top edge. Forelimb retrieval and placement were observed and graded as follows: 0 = immediate and complete placement; 1 = delayed or incomplete placement (> 2 s); and 2 = no placement.

Table 1. Modified neurological severity score

	Points
Motor tests	
Raising mice by the tail	
Forelimb flextion	1
Hindlimb flextion	1
Head moving more than 10 degrees to vertical axis within 30 s	1
Placing mice on the floor (normal = 0; maximum = 3)	
Unable to walk straight	1
Circling toward paretic sides	2
Falling down to paretic sides	3
Beam balancing tests (normal = 0; maximum = 6)	
Grasp of beam side	1
Hugging the beam plus one limb falling down from the beam	2
Hugging the beam plus two limbs falling down from the beam (> 1 min)	3
Attempting to keep balance on the beam but falling down (> 40 s)	4
Attempting to keep balance on the beam but falling down (> 20 s)	5
Falling down without attempt to keep balance or hang onto the beam	6
Sensory tests	
Visual and tactile tests	1
Deep sensory (proprioceptive tests)	1
Absence of the reflexes	
Head shaking when the auditory meatus is touched	1
Eye blinking when the cornea is touched lightly with a cotton tip	1
Motor reflexes in responses to a brief noise from paper snapping	1
Abnormal movements	
Myoclonus, myodystony and seizures	1
Maximum points	18

Elevated body swing test was performed in BPV-treated (n = 13) and saline-treated mice (n = 11) before and at 3, 9 and 13 days after MCAO to evaluate asymmetrical motor behavior (Wang et al., 2009). Mice were held by the tail, the direction of the body swing, defined as an upper body turn of > 10 degrees to either side, was recorded for 30 trials each time. The numbers of left and right turns were counted, and final results were presented as the percentages of turns to the ischemia-impaired side (left side) to 30 (total trials).

Infarct volume assessment

Infarction analysis was performed in BPV- (n = 7) and saline-treated mice (n = 7) at 4 days after MCAO. Briefly, mice were subjected to MCAO followed by daily injection of BPV or saline, starting at 24 h after MCAO. At 4 days after MCAO, mice were decapitated, and brains were harvested and cut into 2-mm-thick coronal sections. Slices were stained in a 1.2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO) and then photographed. Unstained (infarcted) and stained (uninfarcted) areas were analyzed with digital image analysis