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EFFECT OF HIRULOG-LIKE PEPTIDE ON MIDDLE CEREBRAL ARTERY OCCLUSION-INDUCED BRAIN INJURY IN MICE

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Abstract—Hirulog-like peptide (HLP) and low-molecular-weight heparin (LMWH) are thrombin inhibitor peptides. Our previous study demonstrated that HLP could reduce vascular neointimal formation or restenosis in animals undergoing balloon catheter injury in the carotid artery. However, the function of HLP during ischemic stroke is largely unknown. The present study investigated the effect of HLP on brain injury, which was induced by suture of middle cerebral artery occlusion in mice. Mice were divided into four groups, which included a sham group and three treatment groups. Ischemia was induced by transient suture insertion into the middle cerebral artery for 90 min, and mice were either treated with saline, HLP or LMWH. Infarct volume, neurologic deficits and apoptotic factors were measured following 1–14 days of ischemia. We demonstrated that HLP intravenous injection alleviated brain infarct volume and improved neurologic outcomes ($p < 0.05$). HLP decreased levels of protease-activated receptor-1 (PAR-1), caspase-3, malondialdehyde (MDA) and Bcl-2-associated X protein (Bax), increased the activities of catalase and B cell lymphoma-2 (Bcl-2), and improved the ratio of Bcl-2/Bax compared with the control ($p < 0.05$). This study indicates that HLP and LMWH reduced infarct volume and improved neurobehavioral outcomes induced by transient middle cerebral artery occlusion (tMCAO). In addition, HLP had a beneficial effect on the regulation of the thrombin receptor and key apoptosis regulators in the mouse brain. These results suggest that HLP may be a potential alternative therapy for arterial occlusion-induced cerebral ischemia.

Key words: apoptosis, brain, hirulog-like peptide, ischemia, oxidative stress, protease-activated receptor-1.

INTRODUCTION

Thrombin, a multifunctional serine protease generated by the activation of the coagulation cascade, plays a critical role in thrombogenesis, inflammation and atherosclerosis. These effects are reached mainly through protease-activated receptors (PARs). PARs are G protein-coupled proteases that are activated by proteolytic cleavage and generation of a tethered ligand (Schmidlin and Bunnett, 2001). Protease-activated receptor-1 (PAR-1), a major thrombin receptor, is widely distributed throughout the brain, especially in the cortex, hippocampus, and amygdala. PAR-1 is up-regulated after anoxia in hippocampal slice cultures (Striggow et al., 2001). PAR-1 plays a detrimental role during neurite retraction (Turgeon et al., 1998), cell death in hippocampal cultures and motor neurons (Donovan et al., 1997; Turgeon et al., 1999), and potentiation of N-methyl-D-aspartate receptor responses (Gingrich et al., 2000). In addition, it was demonstrated that the infarct volume was attenuated about 3-fold in PAR-1-deficient mice compared with wild-type mice (Junge et al., 2003).

Thrombin plays a major role in ischemic or hemorrhagic injury (Chen et al., 2012). Growing evidence suggests that inhibition of thrombin activity alleviates neuronal damage in brain diseases (Pompili et al., 2011). Inhibiting thrombin activation has been shown to reduce brain damage, ameliorate neurologic deficits and improve animal survival after permanent and transient focal ischemia in rodents (Cuomo et al., 2007). Parenchymal injection of hirudin, aspecific thrombin inhibitor, has

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Abbreviations: APTT, activated partial thromboplastin time; Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma-2; HLP, hirulog-like peptide; LMWH, low-molecular-weight heparin; MDA, malondialdehyde; NSS, neurological severity score; PAR, protease-activated receptor; PBS, phosphate-buffered saline; PFA, paraformaldehyde; ROS, reactive oxygen species; tMCAO, transient middle cerebral artery occlusion.

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been shown to attenuate ischemic brain damage and improve neurological outcomes after middle cerebral artery occlusion (Karabiyikoglu et al., 2004).

Increases in oxidative stress and overproduction of reactive oxygen species (ROS) play important roles in the pathophysiology of ischemic brain injury (Collier et al., 2008). Oxidative stress relates to an imbalance between ROS and the antioxidant system, including vitamin E, vitamin C, β-carotene and scavenger enzymes such as superoxide dismutase (SOD) and catalase (Harris and Amor, 2011; Pamplona and Costantini, 2011; Watson et al., 2012). Oxidative stress causes further damage and may ultimately result in the initiation of pathways that lead to necrotic and apoptotic cell death. Although the mechanism of apoptosis during ischemia has not been well identified, recent studies suggest that caspases and B cell lymphoma-2 (Bcl-2) family proteins contribute to cell apoptosis (Florian-Kujawski et al., 2004; Shuayto et al., 2006). Caspases, such as caspase-3, -6, and -9, are the central molecules involved in the initiation and execution of apoptosis (Shi, 2004). Like caspases, Bcl-2 family proteins are necessary components and essential regulators of apoptotic cell death. These proteins can be grouped into 'Bcl-2 like survival factors' (anti-apoptotic), such as Bcl-2 and Bcl-xL (Gibson et al., 1996; Song et al., 1999; Lalle et al., 2002), and 'Bcl-2 like death factors' (pro-apoptotic), which include Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer 1 (Bak1). The balance between the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax plays a vital role in determining the fate of cells (Wattanapitayakul and Bauer, 2001).

Hirulog-like peptide (HLP), a relatively new thrombin inhibitor peptide, was developed by Dr. Shen's group (Xue et al., 2001). The structure, pharmacokinetics, efficacy and safety of HLP were described in our previous studies (Xue et al., 2001; Tang et al., 2007, 2010). HLP inhibits both the binding of thrombin and the thrombin receptor, which potentially provides more efficient inhibition of thrombin receptor-dependent cellular activities. HLP has a short half-life (25–31 min) compared with low-molecular-weight heparin (LMWH) (112 min), hirudin (72 min) or hirulog-1 (36 min) (Tang et al., 2007), which may explain its relatively less prolongation of bleeding time or activated partial thromboplastin time (APTT) compared with heparin or hirulog-1 in animal models (Xue et al., 2001; Chen et al., 2003; Tang et al., 2010). In previous studies, we demonstrated that HLP prevented balloon catheter dilation-induced neointimal formation in right carotid artery of rats (Xue et al., 2001) and femoral arteries of minipigs (Tang et al., 2010), and carotid artery restenosis in atherosclerotic rabbits (Chen et al., 2003). These effects were associated with its inhibition on injury-induced increases in growth factors, coagulation activators and inflammatory mediators in the vascular wall. Because of the protective effect of HLP on vascular injury and its relative safety, we tested the hypothesis that HLP may be neuroprotective against transient middle cerebral artery occlusion (tMCAO) in mice. We also investigated the mechanism for potential neuroprotective effect of HLP.

EXPERIMENTAL PROCEDURES

Animals and experimental groups

All procedures were performed in accordance with the Care and Use Guide of Laboratory Animals of the National Institutes of Health with the approval of the Scientific Investigation Board of the Shanghai Jiao Tong University, School of Medicine (SYXK-2003-0026). A total of 84 adult male ICR mice weighting between 28 and 32 g were used. Mice were allowed free access to food and tap water and were housed in a temperature-controlled room with a 12-h light–dark cycle.

Mice were divided into four groups: sham group ($n = 7$), HLP group ($n = 7$), LMWH group ($n = 7$) and control group ($n = 7$). HLP was provided by Dr. Shen's lab, and LMWH was obtained from Laboratoire Glaxo Smith Kline (Marly-le-Roi, France). HLP (4 mg/kg), LMWH (3 mg/kg), or control (0.9% (w/v) saline) was intravenously injected at 90 min after tMCAO via the carotid vein. The dose of HLP was 4-mg/kg, which was optimized for reducing infarct size after tMCAO in pilot experiments.

tMCAO

Animals were anesthetized with ketamine (100-mg/kg). During the surgical procedure, rectal temperature was sustained at (37 ± 0.5 °C) with a heating pad (RWD Life Science, Shenzhen, China). Regional cerebral blood flow was monitored using laser Doppler flowmetry (Moor Instruments, Devon, England). Under the operating microscope (Leica, Wetzlar, Germany), the 6-0 suture (Dermalon, 1741-11, Covidien, OH, USA) coated with silicone was introduced into the left external carotid artery (ECA) lumen and gently advanced into the internal carotid (ICA) until it blocked the bifurcating origin of the middle cerebral artery (MCA). After 90 min of tMCAO, blood flow was restored through removing the suture. Cerebral blood flow was reduced by at least 80% during tMCAO (Junge et al., 2003). Sham-operated group mice underwent the same surgical procedure without insertion of the suture.

Behavioral testing

All mice underwent behavioral tests before tMCAO and at 1, 3, 7 and 14 days after tMCAO by an investigator who was blinded to the experimental groups. The series of tests were as follows:

Rotarod test. An accelerating rotarod was used to measure mouse motor function (Chen et al., 2000). The higher score, the better recovery is the motor function. The mice were set on the rotarod cylinder and the time animals remained on the rotarod was measured. The velocity was gradually increased from 4 to 40 rpm within 5 min. The trial was terminated when the mouse fell from the rungs or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rungs. Animals were tested for three trials per day before tMCAO. The mean duration in seconds on the