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RESEARCH PAPER

RIP1K Contributes to Neuronal and Astrocytic Cell Death in Ischemic Stroke via Activating Autophagic-lysosomal Pathway

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Abstract—Although the receptor-interacting protein 1 kinase (RIP1K)-regulated necroptosis can be evoked by cerebral ischemia, the effects of RIP1K in mediating neuronal and astrocytic cell death and the underlying mechanisms remain poorly understood. This study evaluates the contribution of RIP1K to ischemic stroke-induced neuronal and astrocytic cell death, and the activation of autophagic-lysosomal pathway. Using an *in vitro* oxygen and glucose deprivation (OGD) in primary cultured neurons or astrocytes and a permanent middle cerebral artery occlusion (pMCAO) model in rats or mice, we observed the role of RIP1K in the ischemic neuronal and astrocytic cell death and the underlying mechanisms by pharmacological or genetic inhibition of RIP1K. pMCAO or OGD condition led to an increase in RIP1K, RIP3K and RIP1K–RIP3K complex. RIP1K knockdown or necrostatin-1 (Nec-1, a specific inhibitor of RIP1K) treatment reduced infarct volume, improved neurological deficits, increased microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) levels, and attenuated neuronal or astrocytic necrotic cell death in the ischemic cortex. RIP1K knockdown decreased RIP1K–RIP3K complex formation, light chain 3 II (LC3II) and active cathepsin B levels and lysosomal membrane permeability (LMP). Furthermore, a combination of Nec-1 and an inhibitor of autophagy or cathepsin B produced an enhancement of protective effect on neuronal or astrocytic cell death. RIP1K-mediated necroptosis may play important roles in ischemia-induced neuronal and astrocytic cell death through the activation of autophagic-lysosomal pathway.

Key words: ISCHEMIC stroke, neuroprotection, RIP1K, neuron, astrocyte, autophagic-lysosomal pathway.

INTRODUCTION

Ischemic stroke with high morbidity and mortality is the most common type of stroke accounting for more than 80% of all stroke cases. Currently, the tissue plasminogen activator is the only thrombolytic agent approved for acute ischemic stroke by the United States Food and Drug Administration. However, only a small proportion of patients received the thrombolytic treatment mainly because of its narrow therapeutic time window (Zaleska et al., 2009). Brain cell death is a common feature of pathology in ischemic stroke. Although

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Abbreviations: 3-MA, 3-methyladenine; AO, Acridine Orange; DMEM, Dulbecco's Modified Eagle's Medium; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; LC3 II, light chain 3 II; LDH, lactate dehydrogenase; LMP, lysosomal membrane permeability; MAP2, microtubule-associated protein 2; MCA, middle cerebral artery; Nec-1, necrostatin-1; OGD, oxygen and glucose deprivation; pMCAO, permanent middle cerebral artery occlusion; RIP1K, receptor-interacting protein 1 kinase; RIP3K, receptor-interacting protein 3 kinase; scr shRNA, scrambled shRNA; SD, Sprague–Dawley; shRNA, short hairpin RNA; TTC, 2,3,5-triphenyltetrazolium chloride.

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inhibition of apoptosis in animal models of ischemic stroke showed beneficial effects in blocking neuronal and astrocytic cell death, the lack of drug targets in apoptosis pathways has currently prevented the development of apoptotic inhibitors for the treatment of human ischemic stroke (Zhou and Yuan, 2014).

In recent year, a highly regulated form of necrosis, termed regulated necrosis or necroptosis has been discovered and the discovery of necroptosis elicited significant interest in studying the implication of necroptosis in human diseases including ischemic stroke. Necroptosis can be activated by ligands of death receptors such as TNFα, Fas ligand and TRAIL and by a variety of extracellular and intracellular stimuli that lead to the expression of death receptor ligands under apoptotic deficient conditions (Vandenabeele et al., 2010; Zhou and Yuan, 2014). The receptor-interacting protein (RIP) 1 kinase (RIP1K) is a crucial mediator of necroptosis. The activation of RIP1K causes the recruitment of receptor-interacting protein (RIP) 3 kinase (RIP3K), a key downstream mediator of necroptosis. RIP1K interacts with RIP3K to form RIP1K–RIP3K complex (necrosomes) in necroptotic cells (Degterev et al., 2008). RIP1K has three domains, including N-terminal kinase domain, intermediate domain and C-terminal death domain. The N-terminal Ser/Thr kinase domain of RIP1K is required for necroptosis (Holler et al., 2000; Chan et al., 2003; Lin et al., 2004; Degterev et al., 2008). Necrostatins are small molecule inhibitors of RIP1K (Degterev et al., 2005) and necrostatin-1 (Nec-1) is the most commonly used RIP1K inhibitor. The specific inhibition of Nec-1 on necroptosis may be associated with allosterically inhibiting the kinase activity of RIP1K by interacting with the T-loop of the N-terminal kinase domain without affecting other functional domains (Degterev et al., 2008). Previous studies showed that Nec-1 inhibits necroptosis and exhibits neuroprotection in brain trauma, hypoxia, and ischemia/reperfusion injuries (Xu et al., 2010; Rosenbaum et al., 2010; Northington et al., 2011).

Autophagy is a lysosome-dependent intracellular degradation process that functions recycling of cytoplasmic proteins and organelles into bioenergetic and biosynthetic material for maintenance of homeostasis (Nah et al., 2015). Under various stress conditions (Kroemer and Levine, 2008), autophagy is generally considered as one of the cell survival mechanisms. On the other hand, deregulated excessive autophagy can disrupt cellular homeostasis and cause various disorders (Koike et al., 2008; Puyal et al., 2009; Nah et al., 2015). In the context of cerebral ischemia, accumulating evidence indicates that activation of autophagy is involved in the regulation of neuronal cell death, especially at high levels, in different animal models of ischemic brain damage such as hypoxia (Adhami et al., 2006) and global (Wang et al., 2011) and focal ischemia (Wen et al., 2008). Recently, we have found that autophagy is activated in ischemic astrocytes, associating with ischemic astrocytic cell death (Qin et al., 2010; Xu et al., 2014). The increasing evidence has shown that necroptosis and autophagy exist interaction and interplay (Nikoletopoulou et al., 2013). Nec-1 can inhibit the conversion of the microtubule-associated protein LC-3I to LC-3II, a biomarker of autophagy (Degterev et al., 2005, 2008; He et al., 2009; Rosenbaum et al., 2010; Vandenabeele et al., 2010; Dong et al., 2014), suggesting that RIP1K may be involved in the regulation of autophagy activation. Nonetheless, in ischemic stroke, it remains unknown whether RIP1K plays a role in the activation of autophagic-lysosomal signaling pathway in ischemia-induced neuronal and astrocytic cell necroptosis, especially at the lysosomal level.

In the present study, we sought to systematically explore the contribution of RIP1K to ischemic stroke-induced neuronal and astrocytic cell necroptosis and its mechanisms associating with the autophagic-lysosomal pathway activation by pharmacological or genetic inhibition of RIP1K in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley (SD) rats (280–310 g) or male ICR (Institute of Cancer Research) mice (18–22 g) were acquired from the Center for Laboratory Animals, Soochow University (Production license: XCYK-2002-0008). Animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Soochow University, Suzhou, China and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo technique, if available.

Rats or mouse model of permanent middle cerebral artery occlusion (pMCAO)

pMCAO was performed as described (Qin et al., 2010; Xu et al., 2014). Briefly, following anesthesia with 2.5% isoflurane in 100% O_2, holes were made to place laser Doppler flow (LDF) probes for regional cerebral blood flow monitor. A 4–0 (for rats) or 7–0 (for mice) nylon filament was advanced from the right common carotid artery through the internal carotid to the middle cerebral artery (MCA). Rectal temperature was maintained at 37.0 ± 0.5 °C with a temperature-regulated heating pad during the whole procedure. Animals were randomly assigned to different treatment groups (n = 10 for each group), and Nec-1 24 nmol (Sigma, N9037, dissolved in DMSO and diluted to be a 3 mM solution with normal saline) or vehicle was administrated intracerebroventricularly. For 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) staining, brains were sliced into five coronal sections, each 3 mm thick. Sections were stained in 2% TTC in saline for 30 min, and then fixed in 4% paraformaldehyde in PBS, pH 7.4. Infarct volume was assessed with direct method using ImageJ (National Institutes of Health, Bethesda, MD, USA).

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