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Rac1 CONTRIBUTES TO CEREBRAL ISCHEMIA REPERFUSION-INDUCED INJURY IN MICE BY REGULATION OF Notch2

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Abstract—*Background:* Cerebral ischemia–reperfusion (IR) injury is a complex pathological process that can cause irreversible brain damage, neuronal injury or death from brain ischemia. Rac1 GTPase is involved in cellular protection from IR injury. However, the mechanism of protection and the molecules affected by Rac1 remain to be defined. *Methods and results:* C57BL/6 mice were subjected to middle cerebral artery occlusion for 1 h, followed by 24-h reperfusion. In this *in vivo* model of cerebral IR injury, mice treated with the Rac GTPase inhibitor NSC23766 or Rac1 small interfering RNA (siRNA) had better short-term (72 h) neurologic scores, less infarction volume, higher production of antioxidant enzymes, lower lipid peroxide, and reduced apoptosis compared with a vehicle-treated group or a control-siRNA group. However, long-term (14 day) neurologic scores were worse for the two treatments compared to controls. Microarray and quantitative polymerase chain reaction (PCR) revealed that Notch2 was downregulated under NSC23766 treatment. Notch2 protein levels decreased with NSC23766 and Rac1 siRNA *in vitro* and *in vivo*. Cell survival increased with the Notch signaling inhibitor DAPT or Notch2 siRNA and NICD2 attenuated the NSC23766 effect. In addition, immunoblotting showed that DAPT and Notch2 siRNA changed the levels of apoptosis-regulating proteins. NFkB mediated Rac1, which regulated Notch2 in an oxygen glucose deprivation model. Both inhibitors of Notch2 and Rac1 enhanced neural stem cell differentiation. *Conclusions:* This study demonstrated the importance of Rac1 regulation of Notch2 in mediating cerebral IR-induced production of injurious reactive oxygen species and cell death *in vitro* and *in vivo* in the short term. Targeted inhibition of Rac1 or Notch2 is new avenue for *in vivo* therapy aimed at protecting organs at risk from IR injury.

Key words: cerebral ischemia reperfusion, Rac1 GTPase, Notch2, neuron protection.

INTRODUCTION

Cerebral ischemia reperfusion (IR) is a major cause of neuronal injury and functional impairment. IR results in cerebral infarction, which is the leading cause of death and adult disability worldwide (Schaller and Graf, 2004). During IR injury, reactive oxygen species (ROS) are generated, initiating a series of cellular events leading to inflammation, necrosis, and apoptosis, which contribute to tissue injury (Kalogeris et al., 2012). The small GTPase Rac1 has been implicated, along with other intracellular enzymes, in the regulation of ROS generation in many cell types including neurons (Archer and Bar-Sagi, 2002).

Rho-family GTPases are crucial at multiple steps of neural development such as neurite outgrowth and differentiation, axon pathfinding, and dendritic spine formation and maintenance (Sebok et al., 1999; Govek, 2005). Rac1, like other members of the Rho GTPase family, cycles between an inactive (GDP-bound) state and active (GTP-bound) state after a signal is received (Etienne-Manneville and Hall, 2002). Rac1 controls a variety of functions associated with multiple downstream effectors such as PAK and JNKs 1 and 2, which stimulate different signaling cascades (Kaempchen et al., 2003). Previous studies showed that Rac1 is involved in the survival and proliferation of progenitor cells in the subventricular zone of lateral ventricles (V-SVZ) and the neurite outgrowth of V-SVZ-derived neurons (Khodosevich and Monyer, 2010). However, several studies reported opposing findings about neurite formation and neurite outgrowth, possibly due to diversity in animal models, cell types or ages, or organisms used for primary cell studies (Kubo et al., 2002; Fournier et al., 2003; Schwamborn and Puschel, 2004). Rac1 is an important source of ROS in an *in vivo* model of hepatic IR (Ozaki et al., 2000). Zhang et al. reported that inhibition of Rac1 protects hippocampus CA1 from delayed neuronal cell death following global cerebral ischemia (Zhang et al., 2009). Limor et al. showed that Rac1 mediated IR by inducing NADPH oxidase activation and ROS generation, as well as oxidative stress in the CA1 region (Raz et al., 2010). However, the

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Abbreviations: ChIP, Chromatin immunoprecipitation; DCF-DA, Dichlorodihydro-fluorescein diacetate; EDTA, ethylenediaminetetraacetic acid; EMSA, Electrophoretic mobility shift assay; GSH-Px, glutathione peroxidase; IR, ischemia–reperfusion; MDA, malondialdehyde; OGD, oxygen glucose deprivation; PBS, Phosphate-buffered saline; PBST, PBS-tween; PCR, polymerase chain reaction; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; TTC, tetrazolium chloride; TUNEL, triphosphate nick end-labeling; V-SVZ, subventricular zone of lateral ventricles.

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involvement of Rac1 in short-term or long-term cerebral IR-induced cell apoptosis and degradation is unknown.

The expression and activity of Notch2 is induced by ischemic injury in postmitotic neurons (Alberi et al., 2010). Notch2 also regulates morphogenesis of Bergmann glia cells (Estrada et al., 2007) and inhibits granule neuron differentiation in the cerebellum (Solecki et al., 2001). Notch proteins and ligands are present in the central nervous system throughout the lifetime (Presente et al., 2001) and are actively involved in dynamic changes in the nervous system architecture and function. Notch signaling is critically important for organogenesis, regulating many cellular processes essential to development such as self-renewal, differentiation, and apoptosis (Zhou et al., 2010). Notch controls neurogenesis, the growth of axons and dendrites, synaptic plasticity and neuronal death. In this study, we investigated the importance of Rac1 in IR injury and Rac1 regulation through Notch2 in the short-term and long-term response to ischemia in a mouse model of cerebral IR.

EXPERIMENTAL PROCEDURES

Animals

Male, 4-week-old C57BL/6 J mice were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). Before beginning the study, mice were allowed to adapt to the environment for 7 days; healthy mice weighing 19.4 ± 1.8 g were selected for experiments. Mice were housed in an air-conditioned environment with constant temperature and a standard light/dark schedule. All animal experiments were approved by the Institutional Animal Care and Use Committee and Ethics Committee of Harbin Medical University and were in accordance with the guidelines of the Animal Experiment Center of Harbin Medical University. All efforts were made to minimize the number of animals used and their suffering.

Mouse modeling and treatment

Experiments used 5-week-old C57BL/6 J mice, anesthetized with an intraperitoneal injection of chloral hydrate (Fluka Chemie AG, Buchs, Switzerland; 80 g/L in saline solution; 0.4 ml/100 g body weight). As shown in Fig. 1, cerebral ischemia was induced in weight-matched mice by middle cerebral artery occlusion using the intraluminal thread technique described previously (Belayev et al., 1996). Right middle cerebral artery ischemia was maintained for 1 h and blood flow was restored by withdrawal of the thread. Body temperature was maintained around 37 ± 0.5 °C throughout the surgical procedure and for 2 h after the start of reperfusion using a heated surgical platform. Sham controls were performed using the same surgical procedures without artery occlusion. The sham operation group and the IR model group received intracerebroventricular injections of physiological saline at 6, 12, 24, 48, 72 h after surgery.

Rac1 small-interfering RNA (siRNA) was 10-nmol end-phosphorothioated Rac1 antisense oligonucleotides in 10 μL TE buffer (10 mM Tris–HCl, pH 8.0), 1 mM EDTA). The sequence for Rac1 siRNA was 5′-ACTTGATGGCCTG CAT-3′ (Integrated DNA Technologies, Inc., Coralville, IA, USA) and control siRNA was 5′-GTATGGGACTC TACCT-3′. The NSC23766 Rac1 inhibitor (50 mM), control siRNA, and Rac1 siRNA were administered intracerebroventricularly at 6, 12, 24, 48, 72 h after surgery. Animals were assessed for neuroprotection and sacrificed at 72 h. Each group consisted of 10 mice. Brains were dissected to determine biochemical parameters, histopathology and cerebral infarct size.

Neurobehavioral function assessment

Neurobehavioral function was evaluated with Bederson's test and the cerebral infarction volume was observed with tetrazolium chloride (TTC) staining. Neurological behavior was scored at 72 h or 14 d after cerebral IR by a researcher blinded to the experiment according to the modified Bederson's test (Zhang et al., 1997). A score of 0 indicated no observable neurological functional deficit; 1 indicated inability to extend the forepaw fully; 2 indicated decreased resistance to lateral push without circling; 3 indicated movement without clear direction; 4 meant the mouse did not walk spontaneously; and 5 included a depressed level of consciousness. Higher scores indicated greater neurobehavioral dysfunction.

To determine the infarction area, brain tissue was removed, sliced into successive 2.0-mm coronal sections and stained with 2% TTC solution 10 min at 37 °C and fixed in 4% formaldehyde at room temperature overnight (Bederson et al., 1986a; Longa et al., 1989). Infarction areas were quantified using Image J software. Data were expressed as the average infarction area of each group.

Biochemical analyses

Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) in

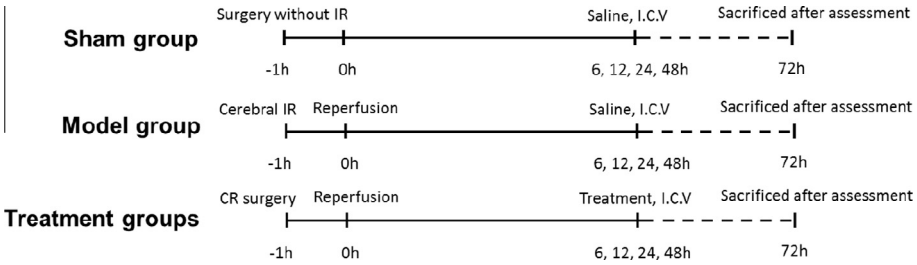

Fig. 1. Schematic representation of the experiments.