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Inhibition of EphA4 signaling after ischemia–reperfusion reduces apoptosis of CA1 pyramidal neurons

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ABSTRACT

Hippocampal CA1 pyramidal neurons are sensitive to ischemic damage. However, the cellular and molecular mechanisms underlying neuronal cell death caused by ischemia–reperfusion (I/R) are not completely clear. Here, we report that the ephrinA/EphA cell–cell interaction signaling pathway plays an important role in the apoptosis of hippocampal CA1 pyramidal neurons induced by I/R. We found that the expression of ephrinA3 and EphA4 is increased in the CA1 region following transient forebrain ischemia. Blocking ephrinA3/EphA4 interaction by EphA4-Fc, an inhibitor of EphA4, attenuated apoptotic neuronal cell death, likely through the inhibition of caspase-3 activation. These results reveal a novel function of ephrin/Eph signaling in the regulation of apoptosis in CA1 pyramidal neurons after I/R.

A restriction in blood supply to the brain leads to ischemic stroke, a debilitating neurological condition that is characterized by a reduction in the oxygen and glucose needed for cellular metabolism. Ischemic stroke is the leading cause of chronic disability and the third leading cause of death in humans [21]. The only effective treatment for ischemic stroke at present is reinstating a blood supply [10]. However, reperfusion often causes tissue injuries when blood supply returns to the brain after a period of ischemia or lack of oxygen. Despite the fact that many studies have focused on ischemic neuronal death, the exact cellular and molecular mechanisms remain unknown [18].

It has been previously documented that CA1 pyramidal neurons in the hippocampus are particularly vulnerable to ischemic insults and undergo cell death several days after transient forebrain ischemia [22]. A number of recent studies suggest that this delayed neuronal death is apoptotic [16]. Apoptosis can be induced by multiple mechanisms, including death receptor activation, radial or chemical exposure and viral infections. One type of apoptotic pathway is mediated by the activation of extracellular signals [26]. Signaling following cell–cell contact provides important information for normal neural function and survival [1]. One prominent example is the binding of Eph receptors with their ephrin ligands. Eph receptors constitute the largest family of receptor tyrosine kinases (RTKs) in mammals and are subdivided into class A and B. EphA and EphB family receptors are activated by ephrinA or ephrinB ligands, respectively. The EphA family members are glycosyl-phosphatidylinositol-linked molecules that bind EphA receptors, while the EphB family members are transmembrane proteins that bind EphB receptors [17].

Most previous studies of the Eph family have focused on nervous system development. Several studies [2,3,19] have suggested that Eph receptors play an important role in the regulation of apoptosis during the development of the central nervous system (CNS). Interestingly, Ephs and ephrins are continuously expressed in the brain during adulthood. However, the function of Eph receptors and their ligands expressed in adulthood remains elusive.

Both EphA4 and ephrinA3 are highly expressed in the hippocampus, particularly in the CA1 region, an area that is highly vulnerable to ischemic insults [20]. The EphA signaling pathway has been reported to regulate the normal function and synaptic plasticity of hippocampal neurons [5]. However, whether Eph receptors are also involved in ischemic injury in the hippocampus has not yet been addressed.

In the present study, we examine the effects of EphA4 signaling on apoptotic neuronal cell death in the hippocampal CA1 region in adult rats. The results suggest that EphA4 signaling plays a novel role after ischemic injury and may provide a therapeutic target for the treatment of stroke.

All experiments were performed on adult male Wistar rats weighing 180–220 g. Food and water were provided ad libitum. Transient forebrain ischemia (15 min) was induced by the use of a modified four-vessel occlusion (4VO) method [22,15]. Briefly, rats

Abbreviations: I/R, ischemia–reperfusion; RTKs, receptor tyrosine kinases; CNS, central nervous system; 4VO, four-vessel occlusion; Cdc42, cell division cycle 42.

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were anesthetized with chloral hydrate (i.p., 35 mg/100 g weight). Both vertebral arteries were electrocauterized permanently, and both common carotid arteries were exposed for subsequent occlusion. On the following day, fully awake rats were restrained, and the carotid clasps were tightened to produce 4VO. Severe transient forebrain ischemia was induced by occluding both common carotid arteries for 15 min. Cerebral blood flow was resumed immediately after release of the carotid artery clasps. The following indicators of forebrain ischemia were monitored: unresponsiveness, loss of righting reflex and catatonic postures. Rats with post-ischemic convulsions were excluded from the study. Rectal temperature was continuously monitored and kept at 37 °C with a heating pad. The animals were randomly assigned to receive daily intracerebroventricular infusions of phosphate-buffered saline or EphA4-Fc (Sigma–Aldrich, Saint Louis, MO, USA; 10 μg/ml) 30 min after reperfusion. Each intracerebroventricular infusion was performed over a 5 min period using a 10 ml syringe through a preimplanted 21 ga cannula in the left ventricle (from the bregma: anteroposterior, 20.8 mm; lateral, 1.5 mm; depth, 3.5 mm).

Seven days after the ischemic insult, the animals were anesthetized with an overdose of chloral hydrate and perfused transcardially with saline followed by buffered 4% paraformaldehyde. The brains were removed and further processed.

The Western blotting assessment of EphA4 and ephrinA3 was performed in control, 6 h and 24 h after I/R groups. Rats were deeply anesthetized and perfused with ice-cold PBS. After decapitation, the brain was rapidly dissected, and coronal slices of dorsal hippocampus (1 mm) were cut with a tissue chopper. The CA1 region was micro-dissected in ice-cold saline. The CA1 tissue was homogenized (in buffer containing 10 mM Tris pH 7.4, 5 mM EDTA, 1% SDS, 0.3 M NaCl), and the material was separated by centrifugation at 14,000 rpm for 30 min. All procedures were performed at low temperature (4 °C). The protein concentration was measured using the Bradford method. Samples in the 2× SDS loading buffer were loaded onto 12% polyacrylamide–SDS gels, and the proteins were separated by electrophoresis. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was then incubated for 2 h at room temperature in blocking medium containing 0.1% Tween 20 and 5% nonfat dried milk. After incubation with primary antibody (Sigma–Aldrich, Saint Louis, MO, USA; rabbit anti EphA4 or ephrinA3, 1:1000 dilutions) in the same buffer for 2 h at room temperature or overnight at 4 °C, the blots were washed three times in Tris for 10 min and incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 h in the same buffer at room temperature. EphA4 or ephrinA3 immunoreactivity was detected by chemiluminescence.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit (Roche, Germany). Brains were paraffin embedded in the hippocampal region (−3.0 to −4.0 mm from the bregma) and were cut into coronal sections (5 μm) for examination. Two pieces of the paraffin-embedded sections were obtained from each rat. Paraffin-embedded hippocampal sections were dewaxed, rehydrated and incubated with Proteinase K working solution for 15 min. After rinsing in an equilibration buffer, the sections were incubated with TdT reaction mixture in a humidified chamber in the dark. The reaction was terminated 1 h later by incubation in a stop buffer at 37 °C for 30 min. The sections were then washed with PBS and incubated for 30 min. After washing with PBS, the sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) for 20 min. The number of neurons in the stratum pyramidal within the CA1 region (400 μm each in length) was analyzed with a fluorescence microscope equipped with the Image Pro Plus System. Determinations of the level of neuronal cell death were made by comparing the number of TUNEL positive neurons with the number of DAPI positive neurons.

Caspase-3 activity was assayed with a Colorimetric Assay Kit (Ac-DEVD-pNA). The hippocampal tissue was incubated with ice-cold lysis buffer (10 μl per mg tissue) and homogenized briefly. The tissue debris was spun down at the maximum speed for 20 min at 4 °C, and the total protein was analyzed by the Bradford assay. The samples were read on a microplate reader at 405 nm.

All data were expressed as the mean ± SEM. The one-way ANOVA was used for statistical analysis. Values of $P < 0.05$ were considered statistically significant.

To test whether EphA4 signaling is involved in hippocampal ischemia, the expression of the EphA4 receptor and its ligand ephrinA3 were first examined by Western blotting in the hippocampus of normal rats as well as rats subjected to 15 min of transient forebrain ischemia. The expression of ephrinA3 in the CA1 region of the hippocampus was upregulated by 245.2 ± 28.4% and 168.75 ± 20.09% at 6 h and 24 h, respectively, and the expression of Eph4 was increased by 309.8 ± 29.5% and 190.34 ± 18.95% at 6 h and 24 h, respectively, after transient forebrain I/R ($n = 10$, $P < 0.05$) (Fig. 1).

To determine whether EphA4 activation is involved in apoptotic neuronal cell death in the CA1 region, the effect of EphA4-Fc, an EphA4 receptor antagonist, on the apoptosis of hippocampal neurons was examined in control animals and in rats subjected to transient forebrain ischemia. In control animals, only a small number (<5%) of the hippocampal neurons underwent apoptosis (Fig. 2A, B, and G). However, apoptotic neurons were significantly increased from 4.62 ± 1.35% to 25.74 ± 1.54% after transient I/R (Fig. 2C, D, and G), which is consistent with the notion that neuronal cell death after ischemia is apoptotic. Interestingly, intracerebroventricular injection of EphA4-Fc dramatically reduced the number of apoptotic neurons in rats that suffered from transient I/R (Fig. 2E–G) but not in control animals ($n = 8$, $P < 0.05$) (Fig. 2G), suggesting that ischemia-induced cell death is likely due to the overexpression of EphA4 receptors.

To further examine whether ischemia-induced apoptosis occurs through the EphA4 signaling pathway, we performed the caspase-3 assay to examine the effects of EphA4-Fc on the activation of caspase-3 in the hippocampal CA1 region. The detection of caspase-3 activation in neurons further supported the above observation. We found that ischemia-induced activation of caspase-3 was attenuated after treatment with EphA4-Fc and inhibition of EphA4 activation (Fig. 3).

In the present study, we found that transient forebrain ischemia significantly increased the number of apoptotic neurons in the hippocampal CA1 brain region, which is likely due to an overexpression of ephrinA3 and EphA4. Inhibition of EphA4 by intracerebroventricular injection of EphA4-Fc (an antagonist of the EphA4 receptor) attenuated caspase-3 activation and apoptotic neuronal cell death, suggesting that EphA signaling is involved in delayed neuronal cell death after I/R.

EphA signaling has been reported to control cell survival and apoptosis of neural projectors during CNS development [2,3,19]. However, less is known about the role of EphA activation in apoptotic cell death in adulthood. In the present study, we found that EphA4 signaling had a direct pro-apoptotic effect on adult CA1 pyramidal neurons after I/R. This notion is supported by three pieces of evidence. First, we found that brief forebrain ischemia significantly increases the expression of EphA4 and ephrin3, which is coincident with the increase of apoptotic hippocampal neurons. Second, ischemia enhances caspase-3 activation, a downstream signaling pathway of apoptosis. Third, both apoptotic neurons and caspase-3 activation are reduced when the Eph4 signaling pathway is blocked by EphA4-Fc, an EphA4 receptor antagonist. These results strongly suggest that EphA4 signaling has a direct pro-apoptotic effect on adult CA1 pyramidal neurons after I/R. A similar result was recently reported in the injured spinal