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Activation of Src tyrosine kinase in microglia in the rat hippocampus following transient forebrain ischemia

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Abstract

To better understand the pathophysiological role of Src protein, a non-receptor protein tyrosine kinase of 60 kDa, in the ischemic brain, we investigated the time course and regional distribution of active Src expression by using a specific antibody against Tyr416 phosphorylated Src (phospho-Src) in the rat hippocampus after transient forebrain ischemia. In the hippocampus of the control animals, active Src expression was too low to be detected by immunolabeling. Beginning 4 h after reperfusion, active Src expression became evident and, after 1 day, had increased preferentially in the CA field of the hippocampus proper and the dentate gyrus. By day 3, active Src expression markedly increased in the pyramidal cell layer of CA1 and the dentate hilar region in temporal correlation with neuronal cell death occurring in these areas, where cells typical of phagocytic microglia showed phospho-Src immunoreactivity. Double-labeling experiments revealed that cells expressing active Src were microglia that stained for biotinylated lectin derived from *Griffonia simplicifolia* (GSI-B_4). Active Src expression began to decline at day 7 and returned to the basal level by day 14 after reperfusion. These results demonstrate increased phosphorylation of Src in activated microglia of the post-ischemic hippocampus, indicating that Src signaling may be involved in the microglial reaction to an ischemic insult.

Keywords: Src; Phosphorylation; Transient forebrain ischemia; Microglia

Src protein, a non-receptor protein tyrosine kinase (PTK) of 60 kDa, is the representative member of the Src kinase family. Cellular Src (c-Src), the homolog of virus Src (v-Src), is widely distributed in many tissues. Dephosphorylation of tyrosine residue 527 (Tyr527) in the C-terminal region may induce its activation and subsequent autophosphorylation of Tyr416 in its kinase region, which further stabilizes its activity [1,12].

Src acts as a common signaling mediator involved in a broad spectrum of physiological responses, including gene transcription, adhesion regulation, and cell differentiation and survival [1]. Recent studies have shown that Src is activated by signals that increase intracellular Ca^{2+} concentration and that Src serves as a signaling protein for pathological mechanisms of neuronal degenerative disease, including ischemia and seizure [11,14]. In vitro kinase assays have shown the ischemia/reperfusion-induced activation of Src [3], and Ma et al. [9] have shown by immunoblotting with antibody against Tyr416 phosphorylated Src that Tyr416 phosphorylation of Src is increased in the rat hippocampus following transient forebrain ischemia. However, the exact cell phenotype involved and the possible role of active c-Src in transient forebrain ischemia remains to be established.

To better understand the pathophysiological role of active Src in the ischemic brain, we assessed the distribution of active Src in the rat hippocampus following transient forebrain ischemia by using a specific antibody against Tyr416 phosphorylated Src.

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Adult male Sprague-Dawley rats (250–300 g) were used in this study. Transient forebrain ischemia was induced by four-vessel occlusion and reperfusion, as previously described by Pulsinelli and Brierley [10] with minor modifications [6]. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). Briefly, the vertebral arteries were electrocauterized and cut completely to abolish circulation. After 24 h, both common carotid arteries were occluded with miniature aneurism clips for 10 min. Only animals with complete electroencephalogram (EEG) flattening upon vascular occlusion were classified as ischemic and used in the study. Rectal temperature was maintained at $37.5 \pm 0.3\,^{\circ}C$ with a heating lamp during and after ischemia. Sham-operated rats with cauterized vertebral arteries and ligatures placed around the carotid arteries were used as controls. No animal convulsed or died following reperfusion or sham operation. Animals were allowed to live for 4 h, 12 h, or 1, 3, 7 or 14 days after reperfusion. Five rats were used for each time-point. Sham-operated animals were treated using the same schedule as the ischemic-reperfused animals. All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996).

At each time point after reperfusion, animals were deeply anesthetized with 16.9% urethane (10 ml/kg) and killed by transcardial perfusion with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Free-floating sections (25 μm thick) were processed for Src immunohistochemistry. After blocking with 10% normal goat serum for 1 h, the sections were incubated with a mouse monoclonal antibody against Tyr416 phosphorylated Src (Calbiochem, San Diego, CA, USA; diluted at 1:100) overnight at 4 °C. Primary antibody binding was visualized using peroxidase-labeled goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA; diluted at 1:200), and 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 as substrate. The specificity of the Src immunoreactivity was confirmed by the absence of an immunohistochemical reaction in sections from which the primary antibody was omitted, or in which it was substituted with non-specific rabbit IgG.

For double-immunofluorescence histochemistry, sections were incubated in a combination of a mouse monoclonal antibody to Tyr416 phosphorylated Src and either a rabbit polyclonal antibody to von Willebrand factor (Sigma, St. Louis, MO, USA; diluted at 1:200) or biotinylated lectin (GSI-B_4) derived from Griffonia simplicifolia (Vector Laboratories Inc., CA, USA; diluted at 1:20) overnight at 4 °C. The sections were then reacted with Cy3-conjugated anti-mouse antibody (Jackson; diluted at 1:500) or FITC-conjugated anti-biotin IgG (Jackson; diluted at 1:50), respectively, for 2 h at room temperature. Control sections were prepared as described above. Slides were viewed using a confocal microscope (MRC-1024, BioRad). Images were converted to TIFF format, and contrast levels of images were adjusted using Adobe Photoshop v. 6.0 (Adobe Systems Inc., San Jose, CA, USA).

Immunohistochemistry demonstrated a low level of basal Tyr416 phosphorylated Src (phospho-Src) immunoreactivity in the sham-operated rat hippocampi at all time points (Fig. 1A and C). Four hours after reperfusion (Fig. 1B), phospho-Src immunoreactivity was augmented in all areas of the hippocampus, and was localized in cells that exhibited the morphological characteristics of activated microglia (Fig. 1D). This expression pattern remained unchanged, but the immunoreactivity was further enhanced after 1 day (Fig. 1E). All immunoreactivity specific to phospho-Src was eliminated when the primary antibody was omitted or substituted with non-specific rabbit IgG (data not shown), confirming the specificity of this antibody.

At day 3 after reperfusion, phospho-Src immunoreactivity was preferentially increased in the pyramidal cell layer of CA1 and the dentate hilar region in temporal correlation with neuronal cell death occurring in these areas (Fig. 1F). In the pyramidal cell layer of CA1, morphological changes in phospho-Src immunoreactive cells—loss of cellular processes and rounding of the cell body typical of phagocytic microglia—were observed (Fig. 1H). At day 7 after reperfusion, increased phospho-Src immunoreactivity was still evident in these regions, but the labeling intensity was decreased (Fig. 1G and I). At day 14, active Src expression had returned to the control level (Fig. 1J).

Comparison with the distribution of GSI-B_4-positive cells in the postischemic hippocampus revealed that the distribution and the density of phospho-Src immunoreactive cells closely correlated with those of activated microglia, identified by their GSI-B_4 labeling (data not shown). To confirm the localization of the phospho-Src in activated microglia, double-labeled immunofluorescence was performed. Co-labeling with phospho-Src and either GSI-B_4 or von Willebrand factor revealed that phospho-Src was localized in GSI-B_4-stained microglial cells (Fig. 2A–C), but not in vascular endothelial cells stained for von Willebrand factor (Fig. 2D–F).

This is the first demonstration of the cellular distribution of phospho-Src in the rat hippocampus following transient forebrain ischemia. Post-ischemic activation of Src, revealed by using an antibody that selectively recognizes tyrosine phosphorylated Src at its major autophosphorylation site, tyrosine 416, was evident within 4 h, reached maximal levels at day 3, and was maintained for at least 7 days. Double-labeling experiments revealed that cells expressing active Src after ischemic injury were activated microglia. Several in vivo studies have recently reported ischemia/reperfusion-induced activation of Src in the rat hippocampus by immunoblotting [9] and in vitro kinase assay [3]. Thus, our data reinforce the idea that Src is activated in the ischemic brain, and provide in situ localization of tyrosine phosphorylated Src in activated microglia.

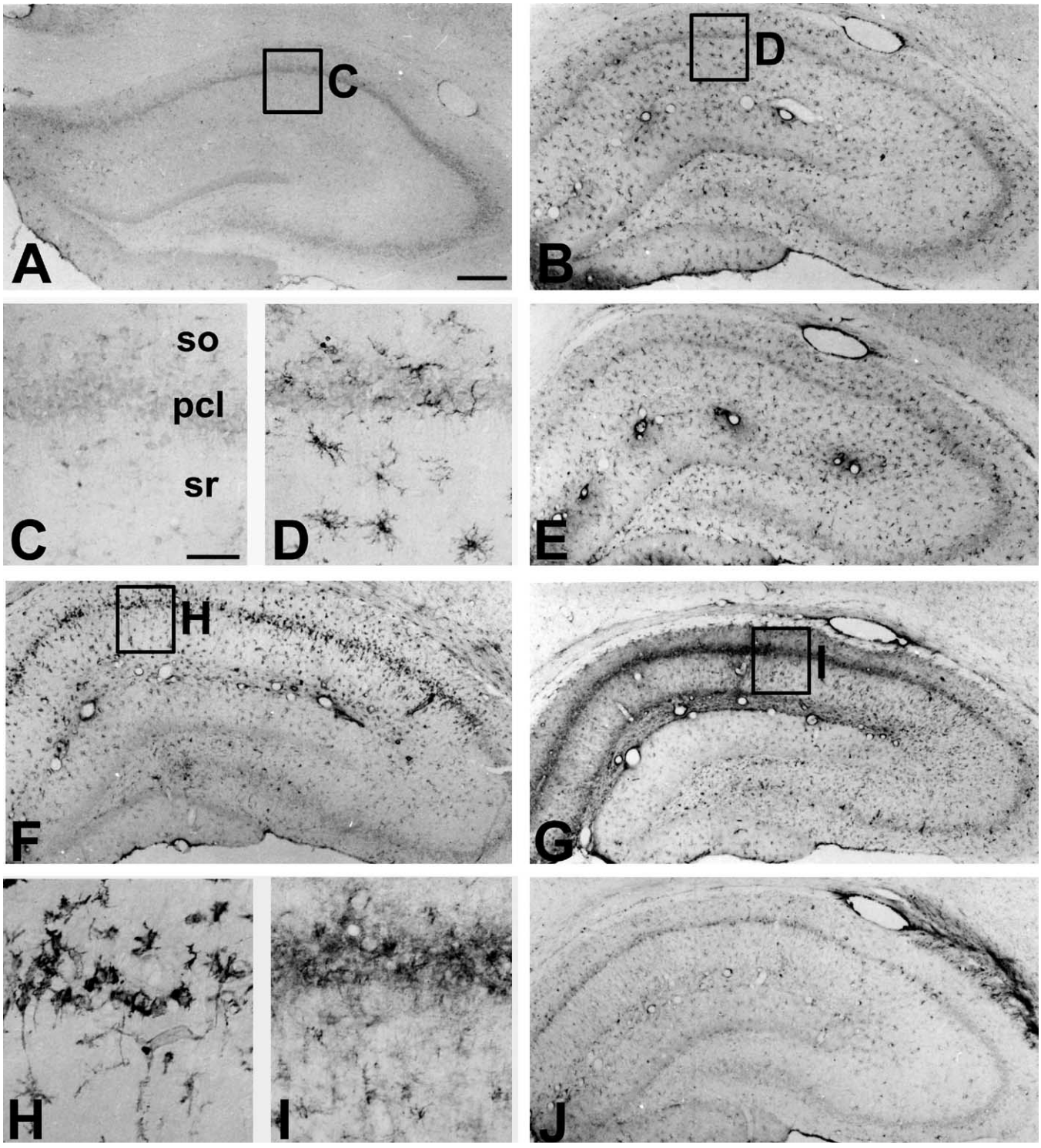

Fig. 1. Changes in phospho-Src immunoreactivity in the rat hippocampus following transient forebrain ischemia. In the control section (A), no significant phospho-Src immunoreactivity was observed in the rat hippocampus. After 4 h of reperfusion (B), phospho-Src immunoreactivity was evident in the dendritic layers of the hippocampus proper and the dentate gyrus. (C and D) Higher magnifications of the boxed areas from A and B, respectively. pcl, the pyramidal cell layer; so, the stratum oriens; sr, the stratum radiatum. After 1 day of reperfusion (E), phospho-Src immunoreactivity had increased rather homogenously over the hippocampus. After 3 days of reperfusion (F), phospho-Src immunoreactivity had increased preferentially in CA1 and the dentate hilar region. By day 7 (G), the expression of phospho-Src had decreased and had returned to the control level by day 14 (J). (H and I) Higher magnifications of the boxed areas from F and G, respectively. Scale bars = 300 μm for A, B, E, F and G; 100 μm for C, D, H and I.

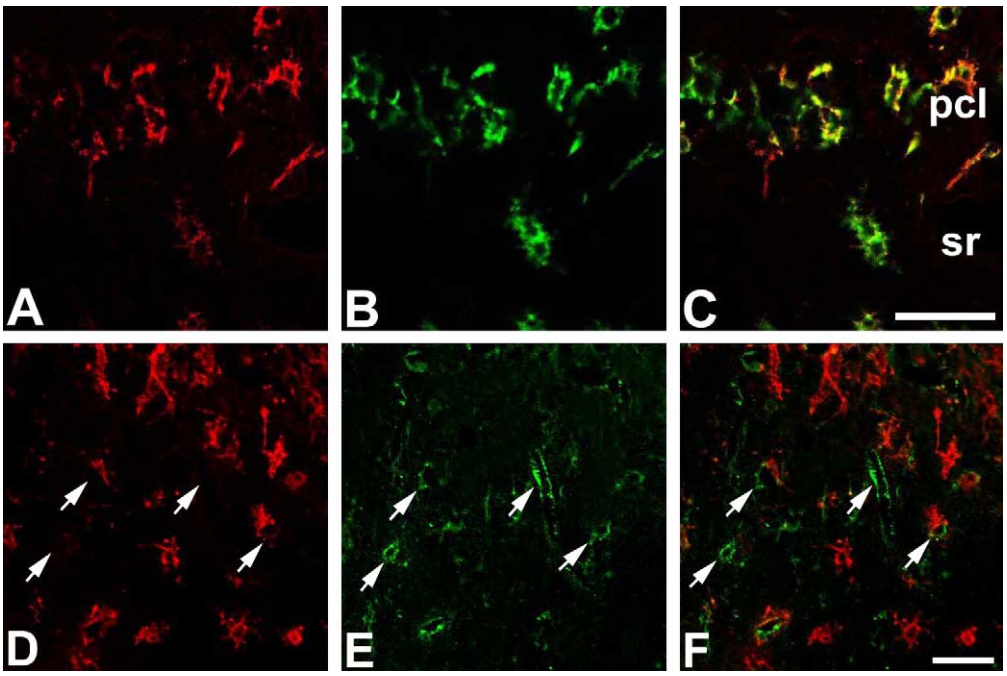

Fig. 2. Identification of phospho-Src in the hippocampus 3 days after forebrain ischemia. Phospho-Src (A, D) was in red, GSI-B_4 (B) and von Willebrand factor (E) in green. (C, F) Superimposed images of Cy3 (A, D) and FITC (B, E). Note that phospho-Src immunoreactivity was colocalized in microglia stained for GSI-B_4, but not in vascular endothelial cells (arrows) stained for von Willebrand factor. pcl, the pyramidal cell layer; sr, the stratum radiatum. Scale bars = 50 μm for A–F.

Active Src expression was accompanied by morphological changes in the microglia. By day 1 after ischemic injury, phospho-Src immunoreactive cells had the morphology of activated microglia, i.e., contraction of their highly ramified processes, and thereby appeared stouter in shape. At day 3, active Src expression was also observed in phagocytic microglia in the pyramidal cell layer of the CA1 region, where severe neuronal death occurred because of the ischemic injury. These results are consistent with the observation that an early generalized microglial reaction in all hippocampal subfields was followed by a degeneration-specific reaction in the areas with neural degeneration after ischemic injury [4].

Recently, enhanced interaction of Src and proline-rich tyrosine kinase 2 (PYK2), another non-receptor tyrosine kinase, with *N*-methyl-D-aspartate (NMDA) receptor subunit 2A (NR2A) has been shown to be induced by ischemia/reperfusion and promote the influx of Ca^{2+} through the NMDA receptor and up regulation of NMDA receptor function [7–9]. It has been reported that tyrosine-phosphorylated PYK2 was observed in microglia in response to CNS insults [2,13]. Tian et al. [13] demonstrated the pronounced and sustained phosphorylation of PYK2 in microglial cells after focal cerebral ischemia and seizure, suggesting that PYK2 is involved in microglial activation as an upstream regulator of p38 mitogen-activated protein kinase (MAPK). Oxidant stress generated during global ischemia/reperfusion activated p38 MAPK in macrophage cell lines through a Src-dependent pathway [5]. Considered together, our data suggest that Src activation, possibly by an interaction with PYK2, may be involved in microglial activation in the ischemic hippocampus. However, the importance of the signaling cascades involving Src in reactive microglia has yet to be clarified.

In conclusion, we have demonstrated that phosphorylation of Src is markedly up regulated in activated microglia in the rat hippocampus after transient forebrain ischemia. These results suggest that Src signaling is involved in microglial activation in response to ischemic injury.

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