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Src REGULATES ANGIOGENIC FACTORS AND VASCULAR PERMEABILITY AFTER FOCAL CEREBRAL ISCHEMIA–REPERFUSION

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Abstract—Developing new strategies to treat cerebral ischemic–reperfusion injury will require a better understanding of the mechanisms that underlie vascular permeability. In this study we examined the temporal expression of Src and angiogenic factors in rat brain after focal cerebral ischemia and reperfusion and analyzed the relationships among those factors. We also investigated the effect of Src inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) in ischemic reperfusion. Rats were subjected to middle cerebral artery occlusion for 90 min followed by reperfusion with or without PP1 treatment. Src mRNA increased at 3 h after reperfusion and then gradually declined. Phosphorylation of Src at Y418 displayed a biphasic increase. Phosphorylation increased as early as 3 h and peaked at 6 h; after decreasing, it peaked again at 3–7 days. Increases in Src mRNA and phosphorylation correlated positively with levels of vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2), and negatively with levels of angiopoietin-1 (Ang-1) and zonula occludens-1 (ZO-1). Changes in the expression of these factors correlated with the progress of vascular permeability, especially early after reperfusion. Hence, dynamic temporal changes in Src Y418 phosphorylation may modulate vascular permeability after cerebral ischemia and reperfusion. PP1 effectively decreased Src Y418 phosphorylation and the expression of VEGF and Ang-2 and increased the expression of Ang-1 and ZO-1. It also reduced cerebral infarct size and neurologic dysfunction. Therefore, Src may represent a new therapeutic target for reducing tissue damage caused by increased vascular permeability.

Key words: angiogenic factors, cerebral ischemia, PP1, Src, vascular permeability, zonula occludens-1.

INTRODUCTION

The Src family comprises a subclass of membrane-associated non-receptor tyrosine kinases that are involved in a variety of cellular signal transduction pathways (Thomas and Brugge, 1997), including gene transcription, adhesion regulation, cell proliferation, and angiogenesis (Brown and Cooper, 1996; Schlessinger, 2000; Theus et al., 2006; Tang et al., 2007). One study has reported that Src kinase activity correlates with vascular endothelial growth factor (VEGF)-mediated vascular permeability and brain damage after permanent focal cerebral ischemia in mice (Paul et al., 2001). Using the same model in rats, we further identified that the expression of Src correlates with VEGF and angiopoietin expression, and with cerebral edema formation (Zan et al., 2011). However, the exact contribution of Src Y418 phosphorylation to vascular permeability after cerebral ischemia and reperfusion remains to be established.

VEGF is an endothelial mitogen as well as a potent mediator of vascular permeability (Schoch et al., 2002). Therefore, it may contribute to brain edema associated with ischemia and reperfusion. The angiopoietins represent another family of proteins that play an essential role in angiogenesis. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) have been well characterized. Ang-1 is required for blood vessel stabilization and maturation and acts as an anti-permeability factor to prevent vascular leakage (Davis et al., 1996; Suri et al., 1996; Risau et al., 1998). In contrast, endogenous Ang-2 serves as a natural inhibitor of Ang-1 activity, disrupting vessel integrity and promoting disassembly of the cellular components (Pfaff et al., 2006). Although these angiogenic factors play critical roles in maintaining vascular integrity, their upstream regulators need to be identified.

To better understand the pathologic role of Src and its possible mechanism of action in cerebral ischemia and

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Abbreviations: Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; BBB, blood–brain barrier; DMSO, dimethyl sulfoxide; EB, Evans blue; MCAO, middle cerebral artery occlusion; MW, molecular weight; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1.

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reperfusion, we characterized the temporal profile of Src mRNA expression and Y418 phosphorylation and the expression of angiogenic factors at mRNA and protein levels after focal cerebral ischemia and reperfusion in rats, and analyzed the possible correlations among them. Additionally, using the same model, we investigated the effect of Src inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) on Src mRNA expression and Y418 phosphorylation, vascular permeability, brain edema formation, lesion volume, and neurologic function.

EXPERIMENTAL PROCEDURES

Animals and ischemia–reperfusion model

Adult male Sprague–Dawley rats weighing 250–300 g were purchased from the Center of Experimental Animals. The rats were housed under standard conditions with free access to rat chow and tap water before and after surgery. All experimental procedures were approved by the Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* techniques.

The rats were randomized into two groups: the sham-operated control group and the ischemia–reperfusion model group. Animals were anesthetized with 10% chloral hydrate (400 mg/kg, i.p.) and then underwent transient middle cerebral artery occlusion (MCAO) with the filament model as previously described (Longa et al., 1989). Body temperature of the rats was continuously monitored and maintained at 37.0 ± 0.5 °C with a heating pad during and after the surgery. Reperfusion was induced after 90 min of ischemia by removing the filament. Rats in the ischemia–reperfusion group were subdivided into seven subgroups based on the duration of reperfusion: 3 h, 6 h, 12 h, 1 day, 3 days, 7 days, or 14 days. At each of these time points after reperfusion, one subgroup of rats was anesthetized; brains were quickly removed after decapitation. Sham-operated rats underwent anesthesia and surgery without the filament insertion.

Treatment with PP1

To address the role of Src in ischemia–reperfusion-induced brain damage and the potential underlying mechanisms, we investigated the effects of the Src inhibitor PP1. PP1 is a cell-permeable pyrazolopyrimidine compound that inhibits Src family tyrosine kinases Lck, Fyn, Hck, and Src (IC_{50} = 5, 6, 20, and 170 nM, respectively). Its molecular weight (MW) is 281.36, and its chemical formula is $C_{16}H_{19}N_5$. PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine), an inactive analog of PP1 (MW = 211.2; $C_{11}H_9N_5$), inhibits the activity of EGFR kinase (IC_{50} = 2.7 μM) and was used as a negative control for PP1. PP1 and PP3 were freshly dissolved in dimethyl sulfoxide (DMSO) and further diluted in phosphate-buffered saline (final dilution, 0.1% DMSO) just before surgery. Rats were randomly assigned to receive PP1 (1.5 mg/kg), PP3 (1.5 mg/kg), or an equal volume of vehicle (0.1% DMSO) by intraperitoneal injection 30 min before surgery (Kusaka et al., 2004). The rats were euthanized by decapitation 1 day after reperfusion.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the ipsilateral cerebral cortex (n = 6/group) by TRIzol Reagent (Invitrogen, Carlsbad, CA). The Transcriptor First Stand cDNA Synthesis Kit (Roche, USA) was used for the reverse transcription reactions. Using 1 μg of total RNA for each sample, we synthesized the cDNA according to the manufacturer's instructions. Then 2 μL of the reverse transcription reaction was used for PCR amplification in a volume of 20 μL. The reactions contained gene-specific primers for Src forward: 5′-AGA GTG CCC TAT CCT GGG AT-3′, SRC reverse: 5′-AAA GTA GTC TTC CAG GAA GGCC-3′; VEGF forward: 5′-CTG CTC TCT TGG GTG CACT-3′, VEGF reverse: 5′-ATA CAC TAT CTC ATC GGG GTA CT-3′; Ang-1 forward: 5′-GAA AAT TAT ACT CAG TGG CTG GAA AAA-3′, Ang-1 reverse: 5′-TTC TAG GAT TTT ATG CTC TAA TAA ACT-3′; Ang-2 forward: 5′-AAA GAG TAC AAA GAG GGC TTC GGG AGC-3′, Ang-2 reverse: 5′-GTA GTA CCA CTT GAT ACC GTT GAA CTT-3′; zonula occludens-1 (ZO-1) forward: 5′-CAG GAA AAT GAC CGA GTC GC-3′, ZO-1 reverse: 5′-CCA ATG TGA CCT TGG TGG GT-3′; or β-actin forward: 5′-CCT CTG AAC CCT AAG GCC AAC-3′, β-actin reverse: 5′-TGC CAC AGG ATT CCA TAC CC-3′. All reactions were carried out in a thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 5 min at 94 °C, 40 cycles of amplification (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s), and 72 °C for 10 min. All products were separated on 2% agarose gels containing 0.5 μg/mL ethidium bromide. Band intensities were evaluated by the Chemidoc XRS imaging densitometer system (Bio-Rad, Hercules, CA) and quantified with Quantity One software.

Western blot analysis

Protein was extracted from ipsilateral cerebral cortex (n = 6/group) with lysis buffer. Equal amounts of total protein extract were loaded onto sodium dodecyl sulfate (10%) polyacrylamide gels and separated by electrophoresis. Protein was then transferred to polyvinylidene difluoride membranes by electroblotting. After being blocked for 2 h with 5% nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20, the membrane was incubated overnight at 4 °C with rabbit polyclonal anti-Src (phospho-Y418, 1:600; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-Ang-2 (1:800; Abcam), rabbit polyclonal anti-ZO-1 (1:600; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-VEGF (1:1000; Abcam), or goat polyclonal anti-Ang-1 (1:800; Santa Cruz Biotechnology). Membranes were then washed and incubated with secondary antibodies for 1 h at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was determined as an internal control.