Src Kinase Inhibition Improves Acute Outcomes After Experimental Intracerebral Hemorrhage

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Background and Purpose—The mechanisms by which intracerebral hemorrhages produce changes of blood flow and metabolism, cell death, and behavioral abnormalities are complex. In this study, we begin to test the hypothesis that intracerebral hemorrhage activates Src kinases that phosphorylate other molecules to produce cell injury and behavioral deficits after intracerebral hemorrhage (ICH).

Methods—ICH was produced in adult Sprague Dawley rats by direct injection of autologous blood (50 μL) into striatum. Src kinase activity, glucose hypermetabolic areas around the ICH, TUNEL-stained cells, and apomorphine-induced rotational behaviors were assessed in animals with ICH pretreated with the Src kinase inhibitor, PP1, or with vehicle.

Results—PP1 (3 mg/kg) blocked increases of Src kinase activity (5-fold) at 3 hours after ICH. PP1 also blocked the areas of glucose hypermetabolism and decreased the numbers of TUNEL-stained cells surrounding the ICH at 24 hours. Finally, apomorphine-induced (1 mg/kg) rotation at 24 hours after ICH was markedly attenuated by previous treatment with PP1 (3 mg/kg intraperitoneal).

Conclusions—PP1 decreases Src kinase activation, glucose metabolic activation, cell death, and behavioral abnormalities after ICH in striatum of adult rats. It is hypothesized that intracerebral hemorrhage, possibly via thrombin activation of protease-activated receptors, activates Src that phosphorylates NMDA receptors, matrix metalloproteinases, and other proteins that mediate injury after ICH. (Stroke. 2007;38:1621-1625.)

Key Words: apoptosis ■ behavior ■ intracerebral hemorrhage ■ Src-kinase ■ striatum

Recent studies of experimental intracerebral hemorrhage (ICH) showed decreased expression of mRNA for some molecules, including Akt and PI3K.1,2 In contrast, expression of genes related to nitric oxide increased including GTP cyclohydrolase (4.1-fold), arginase (4.2-fold), and inducible nitric oxide synthase (NOS2, 15-fold). Importantly for this study, one of the most upregulated genes was a nonreceptor kinase, a Src-family kinase Lyn, that increased expression 21-fold.1,2 Src family kinases may be relevant for the injury that occurs after ICH for a number of reasons. Src potentiates NMDA receptor function through direct phosphorylation of tyrosine residues in the NR2A subunit of the NMDA receptor.3 Thrombin activates Src.4 Finally, Src phosphorylates and regulates a number of molecules including matrix metalloproteinases, HIF, and HIF target genes including vascular endothelial growth factor (VEGF), occluding, and other blood–brain barrier proteins that may be important in producing brain edema and brain injury after ICH.5-17

Clot formation appears to damage the perihematoma brain in part via edema and apoptosis.16,19 These could be mediated by thrombin activation of Src, because thrombin mediates the acute brain edema after ICH.20,21 Src could mediate thrombin-induced edema and injury by Src increases of HIF target genes including VEGF,5,22-24 by Src activation of matrix metalloproteinases,2 and by Src modulation of proteins like occludin in tight junctions of the blood–brain barrier.12,15 The increases of HIF in brain after ICH and thrombin injection25 appear to be mediated by Src,26,27 and HIF induction of VEGF can increase vascular permeability.4 Src activation of matrix metalloproteinases can also increase vascular permeability.5,7,28,29 Based on these data we hypothesize that Src is important for the development of injury, glucose hypermetabolism, edema formation, and apoptosis in peri-hematoma brain.30-33

Therefore, the current study examined the effect of ICH on Src kinase activity; the effect of the Src antagonist PP1 on ICH-induced glucose hypermetabolism; the effect of NMDA antagonist and Src antagonist on TUNEL-stained in peri-hematoma brain; and the effects of the Src antagonist PP1 on behavioral function after acute experimental ICH in rats.

Materials and Methods

Animals

Sprague-Dawley male rats (250 to 300 grams; Charles River Labs, Wilmington, Mass) were used. Generally, there were 6 animals per group for each of the studies described, with 32 animals used for the behavioral studies.
Src Kinase Activity after ICH

Figure 1. Src kinase activity. Adult Sprague Dawley rats were anesthetized and a 50-μL volume of blood or saline was injected into the left striatum. Animals were euthanized 3 hours later. The striatum with the hemorrhage (hemorrhage, 3 hours), the contralateral striatum, and saline-injected striatum were dissected (n=6 separate animals for each group). Some animals had the Src kinase inhibitor PP1 injected intraperitoneally before the hemorrhage (PP1, hemorrhage, 3 hours). Src kinase activity was measured as described in Materials and Methods. The y axis shows Src kinase activity expressed as a fold change from control (1=control levels; 5=5-fold increase in activity). Hemorrhage (hemorrhage, 3 hours) increased Src kinase activity (*P<0.05) compared with the contralateral striatum and the saline-injected striatum; and PP1 blocked this effect (**P<0.05). Hem indicates hemorrhage.

Surgery: ICH Model
ICH was produced by local injection of 50 μL of autologous blood into the striatum of anesthetized adult male rats as previously described.30 The methods are similar to other experimental studies of brain hemorrhage in rodents.34–38

Src Kinase Activity
Three hours after the injection of blood or saline, rats were euthanized and the ipsilateral and contralateral striatum were dissected, homogenized, and protein-isolated. Src protein was immunoprecipitated with a c-Src antibody (Delta Biolabs), which produced a single band on Western blots (not shown). The immunoprecipitated c-Src kinase was assayed for activity using the Tyrosine Kinase Activity Assay Kit (Chemicon). Differences between the 4 groups were assessed with an ANOVA and post hoc Schefe (Figure 1).

Autoradiography and Data Analysis
Local cerebral glucose utilization was measured using [14C]-2-deoxyglucose (1.3 mCi/kg; 55 mCi/mmol; American Radiolabeled Chemicals) 24 hours after ICH as previously described (Figure 2).30

TUNEL Staining
To assess cell death, animals were injected either with vehicle (0.9% saline, intraperitoneal), MK801 (1 mg/kg, intraperitoneal), or with PP1 (3 mg/kg, intraperitoneal) 30 minutes before producing the ICH. After 24 hours animals were euthanized and TUNEL staining was performed on brain sections using methods published from our laboratory30,60 and others.19,31,41–43 For quantification, the TUNEL-stained cells around a hemorrhage were counted using the optical dissector method. Hemorrhage is manifested by hemoglobin/pink/red staining of the striatum. The average number of TUNEL-stained cells was calculated for each animal and differences between the groups assessed with an ANOVA and post hoc Schefe (Figure 3).

Apomorphine-Induced Turning Behavior
Animals had ICH, and had vehicle (0.9% saline), an intermediate dose of PP1 (1.5 mg/kg), or a higher dose PP1 (3 mg/kg) administered intraperitoneally 30 minutes before the ICH. At 24 hours after the ICH surgery, rats were administered apomorphine (1 mg/kg, intraperitoneally). Apomorphine induced significant turning toward the side of the ICH. The numbers of turns per 5-minute interval were counted for each animal over a period of 1 hour (Figure 4). Differences were assessed using a nonparametric Kruskal–Wallis test.

Figure 2. The Src inhibitor PP1 attenuates hypermetabolism in perihematomat brain after ICH. Rats were either pretreated with vehicle (24 hours, hemorrhage; n=6) or pretreated with PP1 (24 hours, hemorrhage+PP1, 3.0 mg/kg; n=6) 15 minutes before injection of blood into the striatum. Twenty-four hours after ICH, the rats were injected with [14C] 2-deoxyglucose and euthanized 30 minutes later by decapitation. The brains were rapidly frozen and sectioned in a cryostat. The sections were dried on a hot plate and the sections were exposed to film for 5 to 7 days. The autoradiographs were scanned and analyzed with MCID elite software. There was a significant reduction in the % hypermetabolic area at all 3 locations from Bregma (1.6, 0.6, and −0.4) for PP1 (24 hours, hemorrhage+PP1) vs saline (24 hours, hemorrhage) and control (*P<0.05 for PP1). Hem indicates hemorrhage.

Figure 3. Decrease in TUNEL positive cells after ICH by pretreatment with the glutamate receptor antagonist, MK801, or the Src kinase inhibitor, PP1. Rats were either pretreated with vehicle (24 hours, hemorrhage) or injected with MK801 (1 mg/kg; 24 hours, hemorrhage+MK801) or PP1 (1.5 mg/kg; 24 hours, hemorrhage+PP1) before injection of blood into the striatum (n=6 per group). The rats were euthanized 24 hours later and the brains were rapidly removed and frozen. The brains were sectioned on a cryostat and the slices were TUNEL-stained. The data represent the averages from 6 animals in each group (*P<0.05 for MK801 or PP1 treatment compared with 24-hour hemorrhage/vehicle). Hem indicates hemorrhage.
Src kinase activity assays showed similar activity in the striatum injected with saline and in striatum contralateral to the blood injection (Figure 1). Injection of unclotted blood into striatum increased Src kinase activity by blocking the vascular permeability effect of VEGF.5 The Src kinase inhibitor PP2 decreased infarct volumes and improved neurological outcomes after middle cerebral artery occlusion.46 In a subarachnoid hemorrhage model, PP1, the same Src family kinase inhibitor used in this study, reduced blood–brain barrier permeability, brain edema, and mortality, and decreased the phosphorylation of VEGF and MAPKs.47 This study concluded that Src contributed to injury after subarachnoid hemorrhage via VEGF and MAPK pathways.47

Our previous study showed increased cerebral glucose utilization at 3 hours and 8 hours after ICH.30 Blocking NMDA or AMPA glutamate receptors blocked increased glucose metabolism.30 These data coupled with studies showing that pp60-Src (Src) potentiates the function of NMDA receptors through direct phosphorylation of the NR2A subunit,13 anchoring NMDA receptors48 and phosphorylation of the scaffolding protein PSD95 at the postsynaptic density of NMDA receptors,49 suggest that blocking Src might block NMDA receptor activation and, in turn, block the ICH-induced increases of glucose metabolism in this study.30 Alternatively, because Src phosphorylates many targets, Src might modulate glucose metabolism after ICH by phosphorylating other molecules, perhaps even on non-neuronal cells.

Previous studies have demonstrated TUNEL-positive cells around ICH.19,31,32,42,50–54 Similar TUNEL-positive cells occur in subarachnoid hemorrhage models.55 The TUNEL-positive cells around ICH can be decreased by caspase inhibitors,19 minocycline,33 memantine,56 FK506,57 knockdown of tumor necrosis factor,58 adenosine A2A activator CGS 21680,59 tauroursodeoxycholic acid,60 15d-prostaglandin J2 that activates PPARγ,54 and erythropoietin.61 The current study adds to this list by showing that glutamate

Discussion

The major finding of this study is that Src kinase inhibition improves several acute outcome measures after experimental ICH. Specifically, ICH markedly activates Src kinase activity, and inhibiting Src kinases decreases areas of glucose hypermetabolism, decreases TUNEL-positive cells around the hemorrhage, and decreases apomorphine-induced turning after ICH. The data are consistent with the hypothesis that Src kinases mediate, at least in part, glucose metabolic changes, cell death, and behavioral deficits after ICH.

Thrombin mediates acute edema in brain after ICH because thrombin inhibitors prevent ICH-induced acute brain edema.37,44 Although thrombin is a serine protease that is essential in the blood coagulation cascade, it also acts on thrombin receptors, called protease activated receptors (PAR), PAR-1, PAR-3, and PAR-4.45 Activation of the PARs leads to activation of specific intracellular kinases, including Src kinases, with several studies showing that thrombin activates Src via PAR thrombin receptors.42 Thus, ICH activation of thrombin at PAR receptors could activate Src kinases.

Src kinases have previously been shown to be of importance in several cerebrovascular diseases. In a mouse model of ischemic stroke, Src inhibition decreased edema and infarct size by blocking the vascular permeability effect of VEGF.5 The Src kinase inhibitor PP2 decreased infarct volumes and improved neurological outcomes after middle cerebral artery occlusion.46 In a subarachnoid hemorrhage model, PP1, the same Src family kinase inhibitor used in this study, reduced blood–brain barrier permeability, brain edema, and mortality, and decreased the phosphorylation of VEGF and MAPKs.47 This study concluded that Src contributed to injury after subarachnoid hemorrhage via VEGF and MAPK pathways.47

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Figure 4. Inhibition of Src kinase activity blocks apomorphine-induced rotational behavior after ICH. Blood was injected into the left striatum of rats. One group of rats was pretreated with saline vehicle (no drug, n=14) and the 2 other groups were pretreated with the Src antagonist PP1 (1.5 and 3.0 mg/kg intraperitoneal, n=9 for each group) 15 minutes before surgery. One day (24 hours) after the surgery, the rats were injected with apomorphine (1 mg/kg) and rotational behavior was measured for 1 hour. Location of the hemorrhage was confirmed by examining brain sections through the striatum after the experiment.
receptor blockade with MK801 and Src kinase blocker can also decrease the TUNEL-positive cells around an ICH.

NMDA receptors are potentiated by thrombin receptor activation that causes Src-mediated phosphorylation of NR2A subunits. NMDA receptors are directly linked to Src via an adapter protein to produce NMDA (NR2a)-NADH dehydrogenase subunit 2-Src complexes. Src kinases regulate glutamate release from synaptic vesicles because PP2 can block glutamate release. Thus, it is not surprising that blocking either Src or NMDA receptors decreases cell death around an ICH. Taken together, these data indicate that Src, glutamate, and inflammatory mediators contribute to cell death around ICH. In these studies, any given compound reduces, but does not eliminate, cell death. It would be of interest to determine whether a combination of agents might eliminate the cell death.

A previous study demonstrated that intrastriatal hemorrhages in adult rats were associated with ipsilateral rotational behavior at 1 to 70 days after high-dose apomorphine. Similarly, administration of high-dose amphetamine also induces rotational behavior with striatal ICH. Both of these studies are consistent with higher dopamine levels and dopamine activation in the intact striatum that produces turning toward the ICH injected striatum. As noted, many neurotransmitter receptors are downregulated in the ICH turning toward the ICH injected striatum. As noted, many neurotransmitter receptors are downregulated in the ICH striatum, including the mRNA for the dopamine D2 receptor. Src kinases regulate glutamate release from synaptic vesicles because PP2 can block glutamate release. Thus, it is not surprising that blocking either Src or NMDA receptors decreases cell death around an ICH. Taken together, these data indicate that Src, glutamate, and inflammatory mediators contribute to cell death around ICH. In these studies, any given compound reduces, but does not eliminate, cell death. It would be of interest to determine whether a combination of agents might eliminate the cell death.

This initial study does not directly address the mechanism by which ICH activates Src kinases. We postulate that ICH-related clotting leads to thrombin activation, which in turn cleaves PAR receptors to activate Src kinases. The thrombin/PAR receptor-mediated Src kinase activation would produce the changes observed in this study. Future studies will be necessary to support the hypothesis that the ICH-mediated changes of Src activation are mediated by thrombin activation of PAR receptors.

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Disclosures
None.

References
13. Liu Y, Zhang G, Gao C, Hou X. NMDA receptor activation results in tyrosine phosphorylation of nmda receptor subunit 2a(n2a) and interaction of pyk2 and Src with n2a after transient cerebral ischemia and reperfusion. Brain Res. 2001;909:51–58.
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