Contribution of Src tyrosine kinase to cerebral vasospasm after subarachnoid hemorrhage

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Object. Mitogen-activated protein kinase (MAPK) has been implicated in cerebral vasospasm after subarachnoid hemorrhage (SAH). This study was conducted to investigate whether Src tyrosine kinase, an upstream regulator of MAPK, is involved in cerebral vasospasm.

Methods. An established canine double-hemorrhage model was used. Twenty-four dogs were divided into four groups: control, vehicle-treated, Src inhibitor PP2–treated, and Src inhibitor damnacanthal–treated groups. Vehicle (dimethyl sulfoxide), PP2, or damnacanthal was injected daily into the cisterna magna of 18 dogs at 3 to 6 days after induction of SAH. Angiography was performed on Day 0 (the day on which the first blood injection was administered to induce SAH) and on Day 7. Western blot analysis of Src and MAPK activation in basilar arteries (BAs) collected on Day 7 post-SAH was performed.

Severe vasospasm was observed in the BAs of vehicle-treated dogs. Mild vasospasm was observed in all dogs treated with Src inhibitors. Phosphorylated Src and MAPK were increased after SAH and activation of these kinases in the BAs was abolished by PP2 and damnacanthal.

Conclusions. The tyrosine kinase Src is an important upstream regulator of MAPK, and inhibition of Src might offer a new therapy in the management of cerebral vasospasm.

KEY WORDS • Src tyrosine kinase • mitogen-activated protein kinase • cerebral vasospasm • dog

Extracellular signal–regulating kinases 1 and 2 are two members of the MAPK family and are known to play an important role in cell proliferation, differentiation, and vasoconstriction. 

During cerebral vasospasm after SAH, spasmogen-mediated ERK1/2 signal transduction contributes to phosphorylation of the actin-binding protein, caldesmon, which removes the inhibitory constraint imposed by caldesmon on the interaction of actin with myosin in smooth-muscle cells, thus leading to a sustained contraction.

Extracellular signal–regulating kinase 1 and 2 are known to be activated by a variety of receptors, including receptor tyrosine kinases and G protein–coupled receptors. It has been suggested that the Src family of tyrosine kinases serve as intermediates for both receptor tyrosine kinases and G protein–coupled receptor pathways to ERK1/2 activation. Nevertheless, Src involvement in this transduction pathway seems to be highly receptor- and cell-type specific. In some cells, lysophosphatidic acid and angiotensin receptors can activate ERK1/2 via a mechanism that involves the tyrosine phosphorylation of Shc proteins by Src family kinases. In other cell types, neither Src nor Shc is required for ERK1/2 activation by these ligands.

In cerebral vasospasm following SAH, we have previously shown that spasmogens such as endothelin or hemo-lysate modulate ERK1/2 activities; however, proximal mediators of vascular ERK1/2 activation by spasmogens have not been fully elucidated, and the mechanisms responsible for elevation of ERK1/2 associated with cerebral vasospasm are unclear. We propose that nonreceptor tyrosine kinases of the Src family are important upstream regulators of ERK1/2 and that alterations in Src activation can underlie the abnormal signaling by spasmogens that is present in cerebral vasospasm after SAH. This is based on findings that Src is expressed in vascular smooth-muscle cells.
and that the Src family of protein tyrosine kinases, which characteristically interacts with transmembrane tyrosine kinase receptors, also interacts functionally with G protein–coupled receptors. Downstream targets of Src include p21Ras, which activates MEK1/2 and, in turn, phosphorylates ERK1/2. Many tyrosine kinases of the Src family have been identified. Of these, the 60-kD c-Src is the prototype.

To our knowledge, there are few data regarding the role of Src in the spasmogen-mediated ERK1/2 signaling that is present in cerebral vasospasm following SAH. In the present study, we report that Src and ERK1/2 are activated after SAH and that Src inhibitors suppress angiographically verified cerebral vasospasm and both Src and MAPK activation in a canine double-hemorrhage model. We, therefore, suggested that the activation of Src tyrosine kinases may represent a key element in the mediation of cerebral vasospasm and that they appear to be activated upstream of ERK1/2.

Materials and Methods

This protocol was evaluated and approved by the Animal and Ethics Review Committees at the University of Mississippi Medical Center and by the Animal Care and Use Committee at the Louisiana State University Health Sciences Center.

Inhibitors of Src

Commercial preparations of damnacanthal and PP2 were obtained from BIOMOL Research Laboratories Inc., Plymouth Meeting, PA. Both drugs were diluted to 10 mM in DMSO.

Experimental SAH Model

Twenty-four adult mongrel dogs of either sex, each weighing 20 to 25 kg, were used either for the double-hemorrhage model or for the normal control group. Dogs chosen for the SAH model were anesthetized with 10 mg/kg thiopental and mechanically ventilated during the experiments. Their body temperatures were kept at 37°C with the aid of a heating blanket; their mean arterial blood pressure and blood gas levels were monitored through a catheter inserted into the FA and maintained within normal ranges. Subarachnoid hemorrhage was induced according to the method of Varsos, et al., as described previously. For cerebral angiography, a left vertebral artery was catheterized with a No. 4 French catheter via the FA. A baseline vertebrobasilar artery angiogram was obtained. The cisterna magna was punctured transfundicularly and 0.4 ml/kg of CSF was withdrawn. An equivalent amount of arterial blood was withdrawn from the FA and immediately and slowly injected into the cisterna magna. The dogs were then placed prone and tilted at a 20° angle with their heads down for 10 minutes to permit pooling of blood around the BA.

The day on which the first blood injection was given was considered Day 0. On Day 2, the same blood injection procedure was repeated without performing angiography.

Before the procedures the dogs had been randomly divided into four groups. In one group of dogs (six animals) SAH was not induced and the animals were treated with vehicle (DMSO), damnacanthal, or PP2. Treatment began on Day 0 and ended on Day 6. Vehicle or drug was administered by intracisternal injection; the first injection was given 1 hour after the second blood injection.

Approximately 40 μl of damnacanthal, PP2, or DMSO was diluted with 1 ml of CSF and injected into the cisterna magna. The doses of the inhibitors were individually calculated for each animal to reach similar drug levels in the dogs’ CSF. The calculations took into account the relative size of the CSF space to obtain the same final concentration of drug or vehicle in CSF, assuming that the canine CSF volume is 2 ml/kg. Angiography was repeated on Day 7, after which the dogs were killed by an overdose of pentobarbital (120 mg/kg).

The dogs’ brains were removed within 10 minutes after death. Guided by a surgical microscope, we carefully removed the BAs from the brainstem. The BAs were immediately frozen in liquid nitrogen and stored at −80°C until used for Western blot analysis.

Measurements of Arterial Diameter

Arterial diameters were measured in a double-blind fashion by examining magnified angiograms. To eliminate magnification differences on the angiograms, a radiodetectable scale was placed on the dog’s chin during the angiography session. The same scale was always used and placed at the same site on the dog’s chin. Using the size of this scale as a standard, all diametric values of arteries were adjusted relative to the scale. Two researchers independently measured the diameters of the arteries on the magnified angiograms at three points: distal (just before the bifurcating superior cerebellar arteries), proximal (just after the vertebral artery union), and central (the midpoint between the previous points) portions of the BA. The mean of these three measurements was calculated to yield the diameter of the artery. The mean of the values measured in both studies was taken as the final diameter of the BA. The caliber of the BA on Day 7 was calculated as the percentage of the mean diameter of the BA on Day 0 before blood injection in each dog.

Western Blot Analysis

The method used for the Western blot analysis of canine BAs has been described previously. The frozen arteries were placed in 200 μl of an extraction buffer containing 50 mMol/L Tris-HCl (pH 7.6), 1% nonylphenol ethoxylate (Igepal), 0.25% sodium deoxycholate; 150 mMol/L NaCl, 1 mMol/L, ethylenediamine tetraacetic acid, 1 mMol/L phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mMol/L NaVO₃, and 1 mMol/L NaF; and were homogenized for 20 minutes at 4°C by applying an ultrasonic wave (10 seconds, three times). The insoluble material was removed by centrifugation at 16,000 G at 4°C for 15 minutes. The samples (20 μg protein) were separated by performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a 10% polyacrylamide gel. After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk in TBST. The membranes were washed and incubated with the primary antibodies at 4°C by using 1% nonfat milk in TBST.

The following primary antibodies were obtained from commercial sources: goat polyclonal anti-Src phosphospecific antibody and goat polyclonal anti-ERK1/2 phosphorylated antibody (Biosource International and Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) and rabbit polyclonal anti-ERK1/2 phosphospecific antibody (Biosource International; Camarillo, CA); these two antibodies can detect phosphorylated forms of proteins and have been used to represent kinase activity or activation previously by others. Accordingly, we used either phosphorolyzed Src–MAPK or Src–MAPK activation in this study. Following incubation with the primary antibodies, the nitrocellulose membranes were washed with TBST and incubated with the appropriate horseradish peroxidase–labeled secondary antibodies (Biosource International and Santa Cruz Biotechnology) by using 1% nonfat milk in TBST for 1 hour at room temperature. An enhanced chemiluminescence system (Amersham; Buckinghamshire, UK) was used to visualize the protein bands. The results were quantified using Quantity One Software (BioRad; Hercules, CA).

Clinical Assessment

The dogs were divided into three classes according to the following criteria: Class 1, the dog was active, had a normal appetite, and exhibited no focal neurological changes; Class 2, the dog was not active, had a poor appetite, or was somnolent; and Class 3, the dog exhibited focal neurological changes (ataxia or paresis). All scores were recorded based on the independent observations of a veterinarian, who was blinded to the study methodology, and one of the researchers. Scores recorded on Day 6 (1 day before the dogs were...
killed) were used and contained the following components: appetite (normal appetite = 0; decreased appetite = 1); activity (dog is active [barking and wagging tail] = 0; dog is not active = 1); neurological deficit (no deficit = 0; apparent deficit [paresis, ataxia] = 1).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences between the control and other groups were compared by one-way ANOVA and then by the Tukey–Kramer multiple comparison procedure if a significant difference had been determined by ANOVA. The clinical scores were compared by Kruskal–Wallis one-way ANOVA on Ranks, and then, if significant differences were found, by the Dunn multiple comparison procedure. A probability value less than 0.05 was considered statistically significant.

Results

Vehicle-Treated Group

All dogs in this group developed severe vasospasm, as shown by angiography (Fig. 1). The mean value of the residual diameter of the BA on Day 7, as a percentage of that on Day 0, was 42.6 ± 1.5 (Fig. 2). The dogs were very sick and had a mean clinical score of 2.17 (Fig. 3), resulting from decreased activity, poor appetite, and neurological deficits.

Inhibitor-Treated Groups

In the PP2-treated group and the damnacanthal-treated group, mild to moderate vasospasm (Fig. 1) was observed in all dogs. The mean residual diameters of the BAs on Day 7, as a percentage of that on Day 0, were 83.2 ± 2.9% and 75.7 ± 4.4%, respectively (Fig. 2). The diameters in these groups were significantly less than that of the DMSO-treated group (p < 0.05, ANOVA). No statistical difference in the diameter of the BAs was noted in the two treatment groups (p > 0.05, ANOVA).

Most of the dogs maintained a good appetite, moderate activity levels, and had no focal deficits. The mean clinical scores of the PP2-treated group and the damnacanthal-treated group were 0.92 and 1.5, respectively. Even though scores in both inhibitor groups were better than those of the DMSO-treated group (2.17), only the scores of the PP2-treated dogs were statistically improved (p < 0.05, Kruskal–Wallis one-way ANOVA on Ranks; Fig. 3). No significant difference was noticed between the clinical scores of these two treatment groups (p > 0.05, Kruskal–Wallis one-way ANOVA on Ranks).
Western Blot Analysis

Activation of Src and MAPK in healthy dogs was used as a 100% standard with which to compare each activation in the groups in which SAH was induced. In the DMSO-treated group, Src and MAPK activation was significantly enhanced by SAH on Day 7 (149.8 and 156.6%, respectively) compared with that in the BAs of healthy dogs without SAH ($p < 0.05$ compared with the vehicle-treated group, ANOVA; Figs. 4 and 5). Treatment with PP2 significantly suppressed the activation of Src and MAPK compared with the DMSO-treated group ($p = 0.004$ and $p = 0.0039$, respectively, ANOVA). No difference was noted between the control group and the PP2-treated or damnacanthal-treated group ($p > 0.05$, ANOVA).

Discussion

In the present study we have demonstrated the following points. 1) Severe cerebral vasospasm occurred after double hemorrhage on Day 7 and affected the clinical outcomes of dogs (poor clinical scores). 2) Phosphorylated Src and MAPK were increased in canine spastic BAs on Day 7 after experimental SAH. 3) Intracisternal administration of an Src inhibitor, PP2 or damnacanthal, abolished the activation of Src and MAPK. 4) These inhibitors attenuated angiographically measured vasospasm and improved clinical scores.

The signal transduction pathways in cerebral vasospasm after SAH remain controversial. The specific members of tyrosine kinase relating to triggering or mediating prolonged smooth-muscle contraction have not been fully elucidated. The Src family–selective tyrosine kinase inhibitors, PP2 and damnacanthal, have been used as powerful tools in...
the investigation of the molecular and cellular mechanisms of physiological and pathophysiological events associated with the activation of the Src family of tyrosine kinases. The drug PP2 is known to inhibit the Src family of tyrosine kinases at submicromolar concentrations in vitro and at low micromolar concentrations in intact cells without affecting other protein tyrosine kinase and receptor tyrosine kinase activities. Damnacanthal is also known to be an Src family inhibitor and was used in one of our previous studies. In the present study, when PP2 and damnacanthal were injected into the cisterna magna, they abolished the enhanced Src and MAPK activation due to experimental SAH in the BA on Day 7 and reduced vasospasm. These results suggest that Src-dependent MAPK pathways contribute to cerebral vasospasm after SAH. To our knowledge, this is the first study in which it has been demonstrated that administering selective Src kinase inhibitors reduces cerebral vasospasm in the canine double-hemorrhage model.

At least nine members of the Src family of tyrosine kinases (Fyn, Yrk, Fgr, Yes, Src, Lyn, Hck, Lck, and Blk) have been identified in various mammalian tissues. Because PP2 and damnacanthal inhibit most of these members of the Src family, simply evaluating the effect of Src inhibitors cannot confirm which Src kinase in this family could influence contractility the most and lead to cerebral vasospasm. It has been established that pp60c-Src is the major isoform expressed in the vasculature and of all the isoforms, pp60c-Src is the one that is activated in angiotensin II–induced vascular smooth-muscle contraction. We speculated that the inhibitory effect of PP2 and damnacanthal on cerebral vasospasm is most likely conducted through pp60c-Src inhibition. This speculation is confirmed by our data (Fig. 4), which show that a specific antibody for pp60c-Src detected an elevation of pp60c-Src at 7 days after experimental SAH and that Src inhibitors reduced the enhancement of pp60c-Src in the spastic BA. Thus, activation of pp60c-Src might lead to MAPK activation and cause prolonged contraction of cerebral arteries in this canine double-hemorrhage model.

Receptor Activation and Src Signaling in Cerebral Vasospasm

As a group of serine/threonine kinases that are activated by a cascade of protein kinases to induce biological responses, MAPK is involved in smooth-muscle contraction. Key components of the ERK pathway include the small GTPase Ras, the cytosolic serine/threonine kinase Raf-1 (MAPK kinase kinase), and MEK1/2 (MAPK kinase), which phosphorylates and activates ERK1/2 (Fig. 6). After SAH, spasmogens activate either growth factor receptors or G protein–coupled receptors, which leads to the activation of Src, Ras, Rho, and/or other tyrosine kinases.
bral vasospasm. Inhibition of different growth factors and G protein–coupled receptor agonists, such as endothelin, in bloody clots and in blood clots 3.7,20,21 have been documented during cerebral vasospasm. The tyrosine kinase Src might play a key role in mediating the signals from the activation of receptors to the activation of MAPK during cerebral vasospasm.

In previous studies, activation of MAPK pathways has been attributed to the activity of growth factor receptors. Binding of growth factors, such as endothelial growth factor or platelet-derived growth factor, to receptor tyrosine kinase leads to receptor dimerization and autophosphorylation. Activated receptor tyrosine kinases lead to Src activation, 6,15,16,27 which is assisted by Grb2. Binding of Grb2 (either directly or via other adaptor proteins such as Shc) to activated growth factor receptors promotes the translocation of the Grb2-associated protein, SOS, to the membrane. The guanine nucleotide exchange factor SOS may catalyze the activation of Ras directly and/or via Src activation by promoting the GTP–guanosine diphosphate exchange. The Ras protein is well known to activate Raf-1, which activates MEK1/2 and, finally, results in stimulation of ERK1/2. 2

Src activation can phosphorylate cytosolic substrates such as caldesmon, leading to prolonged smooth-muscle contraction. On the other hand, in G protein–coupled receptors, all four G protein subfamilies (Gαq, Gαi, Gαs, and Gα12/13) have been implicated in the activation of the MAPK cascade. 37

Activation of these receptors leads to the stimulation of phospholipase C and the formation of diacylglycerol and inositol 1,4,5-triphosphate involves intracellular Ca2+ mobilization, which activates Ca2+-dependent Pyk-2 kinase. In concert with Grb2/SOS complexes, Src acts to mediate Pyk2-induced activation of specific MAPK cascades. 37 Therefore, in MAPK pathways that have originated either from protein tyrosine kinase or from G protein–coupled receptors, Src may play an important role in activating ERK1/2 via a small GTPase Ras, associating with the complex of Shc-Grb2-SOS. The aforementioned pathways for Src and MAPK activation are shown schematically in Fig. 6.

Nonreceptor Activation and Src Signaling in Cerebral Vasospasm

Cerebral vasospasm is caused by multiple factors including both receptor agonists and nonreceptor stimulators. The main component of blood clots is hemoglobin—oxyhemoglobin, deoxyhemoglobin, or methemoglobin. Hemoglobin breaks down into different metabolites, such as bilirubin, heme and heme, globin, and free Fe2+. All these end products of hemoglobin have been shown to play a role in cerebral vasospasm. 39 Oxidation of hemoglobin generates free radicals, which lead to lipid peroxidation and cell damage. Both free radicals and lipid peroxidation contribute to smooth-muscle contraction and have been suggested to be important factors for cerebral vasospasm. 39 One of the actions of free radicals is to increase intracellular Ca2+ and activate PKC. 39

Some investigators have reported that Src also modulates cytosolic Ca2+, which is important in the activation of ERK1/2. 61,65,66 The inhibitor of Src has been shown to reduce cytosolic Ca2+ directly. 5 Furthermore, various isoforms of PKC are capable of directly activating Raf-1 or phosphorylating calpain. 24,53 Some investigators have reported that Src also modulates cytosolic Ca2+, which is important in the activation of ERK1/2. 61,65,66 The inhibitor of Src has been shown to reduce cytosolic Ca2+ directly. 5 Furthermore, various isoforms of PKC are capable of directly activating Raf-1 or phosphorylating calpain. 24,53

Conclusions

On the basis of our data we can infer that nonreceptor tyrosine kinase Src plays an important role as an upstream effector of MAPK in prolonged smooth-muscle contraction. The findings of this study raise the possibility that Src family inhibitors may be candidates for use in treatment of cerebral vasospasm after SAH. Further studies to evaluate the toxicity, half life, and blood–brain barrier permeability are warranted.

References


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