Suramin-induced reversal of chronic cerebral vasospasm in experimental subarachnoid hemorrhage

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Object. The naphthylsulfonate derivative suramin is an inhibitor of growth factor receptors (receptor tyrosine kinases) and G protein–coupled P2Y receptors. Both types of these receptors are suspected of being involved in cerebral vasospasm after subarachnoid hemorrhage (SAH). In the current study, the authors examined the therapeutic effects of suramin and a selective P2X-receptor antagonist, pyridoxalphosphate-6-azophenyl-2,4'-disulfonic acid (PPADS), in the reversal of vasospasm in an established canine double-hemorrhage model.

Methods. Twenty-four dogs underwent double blood injection into the cisterna magna, with injections given on Days 0 and 2. The dogs were divided randomly into three groups (six animals in each group) to be treated from Days 2 through 6 with the vehicle dimethyl sulfoxide, suramin, or PPADS. An additional group of six dogs received double blood injection without any treatment and served as an SAH control group. The animals were killed on Day 7. Angiography was performed on Day 0 before blood injection and again on Day 7 before the animals were killed. After the death of the animals, the basilar arteries (BAs) were collected for morphological studies and determination of tyrosine kinase expression, and the bloody cerebrospinal fluid (CSF) produced by the hemorrhages was collected for measurement of oxyhemoglobin and adenosine triphosphate (ATP).

In the SAH control group, the mean diameter of the BAs on Day 7 was 46.23 ± 6.32% of the value on Day 0 (which served as a reference of 100%). In the DMSO-treated group, the mean residual diameter of the BA was 47.77 ± 0.8% on Day 7 compared with the value on Day 0. Suramin, but not PPADS, increased the residual diameter to 74.02 ± 4.24% on Day 7. On Day 7 the level of ATP in the CSF was decreased and the level of oxyhemoglobin was increased, compared with values measured on Day 0. Suramin, but not PPADS, reduced tyrosine phosphorylation in the spastic BAs.

Conclusions. By reducing tyrosine kinase activity, suramin may be useful in the treatment of cerebral vasospasm.

KEY WORDS • P2 receptor • cerebral vasospasm • suramin • tyrosine kinase • dog

Cerebral vasospasm is a major factor contributing to poor outcome in patients suffering from SAH.34,37,99 The main characteristics of cerebral vasospasm are delayed onset, prolonged contraction of major cerebral arteries, and resistance to most known vasodilators.52 Vascular wall structural changes, including tissue proliferation and cell death, are other features of vasospasm.18,38,50,64 The cause of the vasospasm is the clot from the SAH, as well as numerous agonists that activate growth factor receptors (receptor tyrosine kinases) and G protein–coupled receptors.59 Many vasoactive agents, including endothelins and ATPs, cause contraction and vascular wall structural changes by activation of these two types of receptors.4,12,47

Because vasospasm is caused by multiple factors, in the past targeting one or two factors or receptors failed to prevent or reverse vasospasm.37,61 A new therapeutic strategy used in recent years is the targeting of the intracellular molecular signals that mediate multiple extracellular stimuli. Protein kinase C, tyrosine kinase, and MAPK31 have been suggested to be such targets, and related therapeutic strategies have been conducted.15,20,46,53 An ideal candidate should have the ability to inhibit signals from growth factor receptors and G protein–coupled P2Y receptors;27,33,68 however, the possible therapeutic effect of suramin in the reversal of vasospasm has not yet been investigated.

Because suramin is also a selective P2 receptor inhibitor, we selected suramin as well as PPADS, a selective P2X-receptor antagonist, for the current study. Similarly, ATP concentration was measured in the bloody CSF produced by hemorrhage to determine whether the inhibitory effect of suramin is dependent on P2 receptors. In the meantime, the other characteristic of suramin, as an inhibitor of tyrosine kinase, was studied by measuring the phosphorylation of intracellular substrates of tyrosine kinase in the spastic BAs. We also measured levels of oxyhemoglobin in the bloody CSF.

Abbreviations used in this paper: ANOVA = analysis of variance; ATP = adenosine triphosphate; BA = basilar artery; CSF = cerebrospinal fluid; DMSO = dimethyl sulfoxide; IP3 = inositol 1,4,5 triphosphate; MAPK = mitogen-activated protein kinase; PLC = phospholipase C; PPADS = pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; SAH = subarachnoid hemorrhage.
Materials and Methods

Subarachnoid Hemorrhage Model

The study protocol was evaluated and approved by the University of Mississippi Medical Center Animal Care and Use Committee. Twenty-four adult mongrel dogs of either sex, each weighing 18 to 24 kg, were used in this canine double-hemorrhage model. Anesthesia was induced in the dogs by administration of thiopental (10 mg/kg), and the animals received mechanical ventilation. Each dog’s body temperature was maintained at 37°C by applying a heating blanket. A No. 4 French catheter was inserted into the femoral artery to monitor mean arterial blood pressure and blood gases. Experimental SAH was induced according to the method described by Varos, et al., which has been outlined in our previous study. After angiography was performed, 0.4 ml/kg of CSF was withdrawn. An equivalent amount of arterial blood was withdrawn from the femoral artery and immediately injected into the cisterna magna. The withdrawal of CSF and the withdrawal and injection of blood was repeated 2 days later.

Eighteen dogs were separated into three groups of six dogs each, and the animals in each group were treated with vehicle (DMSO), suramin, or PPADS. Dogs were selected randomly for each group and the operators were blinded to the treatment given to each dog. Treatment started on Day 2 postangiography and continued through Day 6. Daily intracisternal injections of vehicle or drug were given to each animal. The first injection was conducted 1 hour after the second blood injection. Both suramin and PPADS were diluted in DMSO to a concentration of 30 mM. Drug solutions (approximately 20 μL, depending on the body weight of the animal) were diluted with 1 ml of the previously withdrawn CSF and injected intracisternally to obtain a final concentration of 30 μM in the CSF of the animal, assuming that CSF volume in dogs is 2 ml/kg. Six additional dogs were not treated and served as an SAH control group. All dogs were killed on Day 7 postangiography. The brain of each dog was removed within 10 minutes after death, and half of the BA was quickly removed, frozen in liquid nitrogen, and stored at −80°C for Western blot analysis. The other half of the BA, which was still attached to the brainstem, was immediately fixed by an injection of 10% buffered formalin. The BA remained fixed in a solution of 10% buffered formalin for 2 weeks before it was carefully removed from the brainstem. The arterial diameter of the BA on magnified angiograms was measured in a double-blinded fashion. Two researchers independently measured the diameter of the artery on the magnified angiograms at three points: the distal, central, and proximal portions of the BA. The mean of these three measurements was calculated to yield the arterial diameter.

Basilar arteries collected from six dogs that were not subjected to experimental SAH comprised a second control group of healthy arteries.

Measurement of ATP and Oxyhemoglobin

Cerebrospinal fluid was collected from the dogs on Day 0 before blood injection. Bloody CSF was collected on Day 2 before the second SAH was induced and on Day 7 immediately after angiography was performed. The CSF was then centrifuged and the supernatant fluids were removed, placed in aliquots, and frozen. The amount of ATP in subarachnoid bloody CSF was measured using an ATP bioluminescent assay kit, as described by Stoodley and colleagues. Concentrations of oxyhemoglobin were measured using spectrophotometry. The absorbance of the samples was measured three times at each wavelength, and the mean value of the measurement was used for calculation of the oxyhemoglobin concentration, as described by both us and others.

Tyrosine Phosphorylation and Western Blot Analysis

The method we used for Western blot analysis of the substrates of tyrosine kinase has been described previously. Briefly, the frozen arteries were homogenized with ultrasonic waves in 100 μl of an extraction buffer at 4°C. The samples (30 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis by using a 7.5% polyacrylamide gel. After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were incubated with antiphosphotyrosine antibody (mouse monoclonal immunoglobulin G) G0a at 4°C. Nitrocellulose membranes were later incubated with a goat anti–mouse immunoglobulin G antibody (horseradish peroxidase conjugate) at room temperature for 1.5 hours. An enhanced chemiluminescence system was used for visualization of the protein bands. The results were quantified using commercially available software.

Statistical Analysis

Data are expressed as the means ± standard errors. Statistical differences between the control group and other groups were compared using one-way ANOVA and by using the Tukey–Kramer multiple comparison procedure if a significant difference was found by ANOVA. A probability value less than 0.05 was considered to be statistically significant.

Sources of Supplies and Equipment

The PPADS was purchased from Calbiochem (La Jolla, CA). The suramin, ATP bioluminescence kit, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The ECL (enhanced chemiluminescence) system was provided by Amersham (Buckinghamshire, UK), and the Quantity One software, used to quantify the results of the Western blot analysis, was acquired from BioRad (Hercules, CA).
Results

Physiological Evaluation

All dogs were healthy before surgery and there was no significant difference in physiological variables (for example, blood pressure, blood gases, and heartbeat) between groups when values were compared on Day 0 and Day 7 (Table 1).

Effects of Suramin and PPADS on Vasospasm

Double blood injection was consistently associated with angiographically demonstrated evidence of vasospasm on Day 7. The mean residual diameter of the BA in animals that received no treatment was 46.23 ± 6.32% of the control value, that is, the diameter of the BA on Day 0. The mean residual diameter of the BA in the DMSO-treated group on Day 7 was 47.78 ± 0.8%, indicating intrathecal injection of DMSO did not alter vessel diameter (p = 0.623, ANOVA; see later paragraph).

The suramin- and PPADS-treated groups were compared with the vehicle (DMSO)-treated group, because these two inhibitors were diluted with DMSO (approximately 20 µl total solution). The mean residual diameter of the BAs in the suramin- and PPADS-treated groups on Day 7 was 74.02 ± 4.24% and 51.25 ± 1.34%, respectively. Figure 1 summarizes BA diameters measured on angiograms obtained on Day 7 in animals in the SAH control and treatment groups as they relate to values obtained on Day 0 before blood injection. Suramin, but not PPADS, significantly attenuated vasospasm (p < 0.001 compared with the SAH control group and the DMSO- and PPADS-treated groups, ANOVA). There were no significant differences among the control SAH group and the DMSO- and PPADS-treated groups (p = 0.623, ANOVA).

Morphological characteristics of BAs in all dogs were studied. In the control SAH group or in DMSO- and PPADS-treated dogs, narrowing of the vessel lumen, severe corrugation of the internal elastic lamina, and the thickness of the vessel wall were observed using light microscopy (data not shown). In suramin-treated dogs, mild-to-moderate narrowing of the vessel lumen and corrugation of the internal elastic lamina were observed. The vessel wall was not markedly thickened (data not shown).

Concentrations of ATP and Oxyhemoglobin in Bloody CSF

The concentrations of ATP and oxyhemoglobin were measured in the CSF collected on Day 0 (18 animals) and the bloody CSF collected on Day 2 (16 animals) and Day 7 (18 animals). Upper: Even though ATP levels decreased on Days 2 and 7, compared with the level measured on Day 0, no statistical significance was achieved (p = 0.136, ANOVA). Lower: Little or no oxyhemoglobin was found in the CSF collected on Day 0. There was a little oxyhemoglobin in some samples, which was probably due to fresh blood contamination. Oxyhemoglobin levels remained at lower micromolar concentrations from Day 2 to Day 7. There are no statistical differences among the untreated and the DMSO-, suramin-, or PPADS-treated groups for CSF collected on Day 7. Not all samples from all animals were used in this study.
suramin-, and PPADS-treated groups). Although the mean levels of ATP in the treatment groups were lower than those measured on Day 0, there was no statistical significance ($p = 0.136$, ANOVA).

The concentration of oxyhemoglobin was measured in CSF collected on Day 0, Day 2, and Day 7 (Fig. 2 lower). The bloody CSF collected on Day 7 was separated into three groups (untreated/DMSO-, suramin-, and PPADS-treated groups). There was little or no oxyhemoglobin in the CSF collected on Day 0. Oxyhemoglobin levels were markedly increased in CSF collected on Day 2 and Day 7 ($p < 0.05$ compared with levels on Day 0, ANOVA). There were no statistical differences between CSF collected on Day 2 and CSF collected on Day 7 from each treatment group ($p = 0.326$, ANOVA).

**Effects of Suramin and PPADS on Tyrosine Phosphorylation**

In samples collected from DMSO-treated animals on Day 7, an increase in the phosphorylation of intracellular substrates of tyrosine kinase was observed in two major ranges, approximately 60 to 79 kD and approximately 116 kD, when compared with normal BAs obtained in control dogs that were not subjected to SAH induction (Fig. 3A). The effects of suramin and PPADS were compared with BAs in control dogs and with DMSO-treated BAs.

When compared with BAs in control dogs, the arteries from the suramin-treated group exhibited a very slight increase in tyrosine phosphorylation at the 60 to 79 kD band, but this increase was definitely lower (although not statistically so) than the increases apparent in the DMSO- and PPADS-treated groups. At the 116 kD band, it was clear that suramin abolished tyrosine phosphorylation. Tyrosine phosphorylation was not significantly reduced by PPADS ($p < 0.05$ compared with arteries in control dogs, ANOVA; Fig. 3B).

When compared with BAs excised from animals treated with DMSO, suramin, but not PPADS, markedly reduced tyrosine phosphorylation at the 116 kD band ($p < 0.05$ compared with the DMSO-treated group, ANOVA). At the 60 to 79 kD band, suramin, but not PPADS, partially reduced tyrosine phosphorylation ($p = 0.066$ compared with the DMSO-treated group, ANOVA; Fig. 3B).

**Discussion**

The observations in this study can be summarized in the following manner. 1) Double blood injection produces several angiographically and histologically verified vasospasms in the BAs of dogs. 2) Treatment with DMSO did not prevent or reverse either angiographically or histologically verified vasospasm. 3) Suramin, an inhibitor of both growth factor and G protein–coupled receptors, attenuated angiographically and histologically verified vasospasm. 4) A selective P2X receptor inhibitor, PPADS, failed to prevent or reverse the vasospasm. 5) The level of ATP in bloody CSF decreased during the progression of vasospasm. 6) The level of oxyhemoglobin in bloody CSF increased along with the time course of vasospasm. 7) Tyrosine phosphorylation increased in the spastic BAs, and suramin, but not PPADS, reduced this tyrosine phosphorylation.

**Comparison of Growth Factor Receptors With G Protein–Coupled Receptors**

The subarachnoid blood clot, including oxyhemoglobin and its breakdown products as well as contractile factors released from the vessel wall, are the causative factors or spasmogens for cerebral vasospasm.1,16,28,40,52,59,67 These spasmogens activate PLC to generate IP$_3$ and diacylglycerol, which in turn activate protein kinase C in cerebral arteries.6,26,31 The tyrosine kinase signal transduction pathway, parallel to the receptor–G protein–PLC–IP$_3$ pathway, plays an important role in the regulation of vascular proliferation and smooth muscle tone.1,14-22 Receptor activation by agonists such as serotonin, norepinephrine, angiotensin II, vasopressin, ATP, and endothelin not only activates PLC to generate IP$_3$ and diacylglycerol, which activates protein kinase C, but also phosphorylates intermediate tyrosine kinases that regulate intracellular Ca$^{2+}$ and smooth muscle contractility.26 Indeed, endothelin-1–induced contraction in the rabbit BA substrates of tyrosine kinase was observed in two major ranges, approximately 60 to 79 kD and approximately 116 kD, when compared with normal BAs obtained in control dogs that were not subjected to SAH induction (Fig. 3A). The effects of suramin and PPADS were compared with BAs in control dogs and with DMSO-treated BAs.

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is mediated by tyrosine kinase and MAPK. Adenosine triphosphate raises intracellular Ca" and activates MAPK in rat basilar smooth muscle cells. Hemolysate activates tyrosine kinase in fibroblasts and Ras in the BA of rabbits. Suramin has the ability to inhibit both receptor tyrosine kinase and G protein–coupled receptor systems, indicating a possible role of suramin in the treatment of vasospasm.

**Suramin: A New Candidate for Vasospasm Treatment**

Suramin is an inhibitor of growth factor receptors, receptor–G protein coupling, and P2X and P2Y receptors. This agent inhibits proliferation, angiogenesis, Ca" elevation, and contraction in vascular systems, including the cerebral vascular systems. This agent has been used in clinical trials of prostate cancer, lymphoma, renal cell cancer, breast cancer, ovarian cancer, and acquired immune deficiency syndrome. The results of these clinical trials have demonstrated that the toxicity of suramin is manageable and reversible. Recently, several clinical trials of new approaches to brain tumor therapy have demonstrated that suramin is well tolerated by patients and exerts antineoplastic activity in high-grade gliomas. It has been reported that suramin decreases the general level of tyrosine phosphorylation by inhibiting growth factor receptors or receptor tyrosine kinase. Additionally, suramin greatly diminishes the downstream factors of the tyrosine kinase pathway, such as MAPK activation. Suramin has been reported to be effective in various vascular diseases; however, the possible effect of this agent in the treatment of vasospasm after SAH has not been examined.

The antivasospasm effect of suramin may be mediated by its inhibitory action on tyrosine kinase. There are several supporting items of evidence in this study. First, suramin, but not PPADS, reduced vasospasm. Even though PPADS is a selective antagonist for P2X receptors, it also has a weak effect on other types of P2 receptors, especially at higher concentrations similar to those used in the current study. Second, the level of ATP was decreased in the bloody CSF that was obtained on Day 2 and Day 7, compared with the level of ATP in the CSF collected on Day 0, which indicates a limited role of extracellular ATP in vasospasm. A similar low level of ATP in CSF was previously reported. Third, suramin, but not PPADS, abolished the enhanced expression of tyrosine kinase in spastic arteries.

There are two clusters of phosphotyrosine that are sensitive to suramin in spastic arteries: those at approximately 60 to 79 kD and 116-kD bands. Other agonists induced similar bands of phosphotyrosine in vascular response. Butcher and associates reported that platelet-derived growth factor and angiostatin II, a G protein–coupled receptor agonist, increased tyrosine phosphorylation in vascular smooth muscle cells. Platelet-derived growth factor–BB stimulation produced two major peaks of protein tyrosine phosphorylation at 75 kD and 180 kD, and angiostatin II stimulation induced two major clusters of protein tyrosine phosphorylation at bands spanning from 60 to 65 kD and 110 to 131 kD. Similarly, Di Salvo and colleagues identified 66-kD and 116-kD tyrosine-phosphorylated proteins in response to stimulation by α1-adrenergic receptors or muscarinic receptors. Those authors commented that the 66-kD and 116-kD tyrosine-phosphorylated proteins play important roles in the vasoconstriction induced by growth factors and G protein–coupled receptor agonists. Because the cause of vasospasm is the blood clot from the SAH, especially oxyhemoglobin within it, the effect of blood components on tyrosine kinase were examined. Oxyhemoglobin induces tyrosine phosphorylation, and the phosphorylated 42-kD and 60-kD proteins were immunologically related to ERK-2 and p60c-Src (c-Src), respectively. Hemolysate produced an increase in the level of tyrosine phosphorylation of two ranges, at approximately 60 to 70 kD and 100 to 120 kD in the aortic smooth muscle cells of rats, fibroblast cells in the BAs of dogs, and bovine pulmonary artery endothelial cells. Thus, the two clusters of tyrosine precipitation bands that responded to suramin in the current study are in the same range of protein tyrosine phosphorylation that has been established in smooth muscle contraction.

In conclusion, suramin reduced angiographically and histologically demonstrated vasospasm in an established canine double-hemorrhage model. The mechanism of suramin is probably related to its action on tyrosine kinase, but not on P2 receptors. Suramin, an agent that has been used in clinical trials for cancer treatment, has potential in the treatment of patients suffering from cerebral vasospasm.

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Suramin-induced reversal of vasospasm


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