Thrombin Reduces Cerebral Arterial Contractions Caused by Cerebrospinal Fluid From Patients With Subarachnoid Hemorrhage

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Background and Purpose—We observed that the second application of cerebrospinal fluid (CSF) from subarachnoid hemorrhage (SAH) patients onto cerebral arterial segments in vitro produces a greater contraction than does the initial application. It was hypothesized that the difference between the first and second applications of SAH CSF was due to the activity of thrombin.

Methods—Canine vertebrobasilar artery was removed under general anesthesia, cut into rings, and suspended in tissue culture baths so as to measure isometric tension. CSF was taken from patients 1 to 3 days after SAH via ventricular drains. CSF was administered in $10^{-2}$ to $10^{-1}$ dilutions. The thrombin antagonist hirudin (5 U) was administered before CSF in some experiments. The arterial tension response to pure oxyhemoglobin ($10^{-4}$ to 3.2 g/dL) and thrombin ($10^{-4}$ to 3.2 U/mL), administered alone or in combination, was also examined.

Results—Hirudin increased arterial tension generated on the initial application of SAH CSF but had no effect on the tension generated by the second application of the SAH CSF, suggesting that thrombin limits the tension generated by vasoconstrictive agents in the CSF. Thrombin and pure oxyhemoglobin administered together produced less tension than that generated in response to oxyhemoglobin administered alone; no additive response was observed by coadministering the 2 vasoconstrictive agents.

Conclusions—In the presence of oxyhemoglobin, thrombin acts to reduce cerebral arterial tension. This interaction between thrombin and hemoglobin may account for the observation that the second application of CSF from SAH patients onto cerebral arterial segments in vitro produces a greater contraction than does the initial application. (Stroke. 2000;31:2149-2156.)

Key Words: hemoglobin ■ plasmin ■ subarachnoid hemorrhage ■ thrombin ■ vasospasm

Recent studies from our laboratory have focused on identifying compounds in the cerebrospinal fluid (CSF) of subarachnoid hemorrhage (SAH) patients that cause contraction of cerebral arteries. These CSF factors are thought to reflect the environment in the subarachnoid clot that contributes to the development of vasospasm. This report describes the importance of thrombin in the generation of cerebral arterial vasoconstriction caused by SAH CSF.

It is generally thought that hemoglobin is the principal spasmogen in the subarachnoid clot.\(^1\) Hemoglobin, particularly oxyhemoglobin, is a vasoconstrictive agent both in vitro\(^2\) and in vivo.\(^3\) Hemoglobin is released during the breakdown of erythrocytes, which begins shortly before the time at which vasospasm develops.\(^4\) Although the hemoglobin concentration in erythrocyte hemolysate correlates well with its vasoconstrictive ability,\(^5\) the hemoglobin concentration in CSF from SAH patients correlates poorly with the ability of the CSF to cause vasoconstriction.\(^6\) The inability of hemoglobin to determine SAH CSF contractility and the disparity in contraction caused by SAH CSF and erythrocyte hemolysate may be due to the presence of vasoactive plasma proteins that would be present in the CSF after SAH.\(^7,8\)

One such protein is the serine protease thrombin. Thrombin has an established role in vasospasm at sites of blood coagulation in coronary arteries.\(^9\) On cerebral arteries, thrombin exerts a biphasic effect, causing transient endothelium-dependent relaxation\(^8\) and more prolonged vasoconstriction by a direct action on smooth muscle cells.\(^10\) Thrombin in the CSF, as measured by the level of fibrinopeptide A\(^11\) or thrombin-antithrombin,\(^12\) is predictive of vasospasm, and serine protease inhibitors that antagonize thrombin reduce vasospasm in a dose-dependent manner after intracisternal injection of whole blood.\(^13,14\) However, a more specific examination of the function of thrombin in SAH vasospasm with the use of selective antagonists has not been performed.

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2149
We became interested in thrombin because of the observation that the first administration of a sample of SAH CSF onto cerebral arterial segments ex vivo produced a smaller increase in tension than did the subsequent administration of the same CSF sample. The data in the present study suggest that the lower tension generated on the initial exposure to SAH CSF is due to thrombin activity, because the initial response could be increased to the level attained by the second SAH CSF exposure if the arterial segment was pretreated with the thrombin antagonist hirudin. Despite its action as a vasoconstrictor, thrombin was also shown to reduce the tension generated by oxyhemoglobin. The ability of thrombin to reduce oxyhemoglobin contractility appears selective, inasmuch as thrombin did not reduce endothelin-1–induced or KCl-induced contractions. No such relation was observed between plasmin and oxyhemoglobin, further indicating the specific nature of the action of thrombin against oxyhemoglobin contraction.

Methods

Animal Handling, Cerebral Artery Harvesting, and Measurement of Cerebral Arterial Tension
All procedures on animals were approved by the Institutional Animal Care and Use Committee. The vertebrabasilar arteries of mongrel dogs were prepared as described elsewhere. Unless otherwise noted, arterial segments were used in only one experiment. Briefly, 4-mm sections of vertebrabasilar artery were cleaned of branches and connective tissue and were hung under 0.75 g of isometric tension by means of stainless-steel hooks passed through the lumen. Artery segments were hung in Krebs’ buffer–filled organ baths that were heated to 37°C and bubbled with 95% oxygen/5% carbon dioxide. During a 1-hour acclimation period, the organ bath buffer was changed every 20 minutes, at which time the resting tension was reset to 0.75 g. After the 1-hour acclimation period, the resting tension was no longer adjusted, and the artery segments were subject to experimental manipulation.

Oxyhemoglobin, thrombin, and plasmin were added directly into the organ bath in amounts that increased the concentration of the compound in the organ bath in half logarithmic steps. A drop of antifoam B was administered to prevent foaming of the solution. The tension generated in response to a certain dose of a compound was measured 3 minutes after administration of that dose. In experiments in which 2 compounds were coadministered, a solution with both compounds was made beforehand that contained sufficient concentrations of each compound so that the volume of solution administered into the organ bath to obtain a given concentration of each compound was not different from the volume that would have been administered if each compound had been applied separately.

Acquisition and Preparation of Human SAH CSF Samples
All procedures involving humans were carried out under protocols approved by the Institutional Review Board. Samples of CSF from patients with SAH were obtained in cases with radiological evidence of SAH and a known date of hemorrhage onset. In such patients, CSF samples were drained from a ventricular catheter into a reservoir that was emptied by the nursing staff every 8 hours. CSF samples were collected from the reservoir on either posthemorrhage day 1 (n = 2 samples) or on posthemorrhage day 3 (n = 3 samples). Each sample was taken from a different patient, and no patient was treated with fibrinolytic compounds. CSF samples were individually stored without any treatment at −80°C and were thawed immediately before use. CSF samples were diluted in Krebs’ buffer for use in dose–response experiments. Each sample was individually applied to at least 2 separate cerebral artery segments.

Acquisition and Preparation of Monkey Subarachnoid Blood Clot
Subarachnoid Blood Clot

Measurement of Hemoglobin in Human SAH CSF Samples and Monkey Subarachnoid Blood Clot

The hemoglobin content of CSF samples from patients with SAH or of subarachnoid clot extracts from monkeys was determined by the characteristic absorbencies of hemoglobin at 577 and 630 nm (A 577nm and A 630nm, respectively). The amounts of hemoglobin in ferrous and ferric forms (in μmol/L) were then calculated with the following equations:

\[
\text{[oxyhemoglobin]} = \frac{(66 \cdot A_{577nm}) - (80 \cdot A_{630nm})}{4}
\]
human hemoglobin was supplied at a concentration of 10 g/dL in Ringer’s lactate buffer.

Statistical Analysis
Determination of the response of cerebral artery segments to a treatment that involves multiple doses or comparison of the response of the cerebral artery segments to 2 different treatments, each involving multiple doses, was made by ANOVA. ANOVA results are reported as F and probability values. Post hoc analysis was performed with the Tukey methodology. Comparison of the effects of a single treatment (ie, hirudin treatment) on cerebral artery segment tension was performed with repeated-measures t tests. The t test results are reported as r and probability values. A value of P<0.05 was considered statistically significant.

Results
Hemoglobin Content and Serine Protease Activity of Human SAH CSF Samples
The hemoglobin content of the CSF samples from patients with SAH was measured by spectrophotometry. For the samples (n=5) that were used in cerebral artery tension experiments, the following values were obtained: 27±11 μmol/L oxyhemoglobin and 18±5 μmol/L methemoglobin.

The amount of serine protease activity in the SAH CSF samples was also measured. Specific thrombin activity in the CSF samples was quantified by inhibiting the thrombin in the solution with excess hirudin. The average total protease activity in the CSF samples was found to be 0.014±0.011 U/mL. Only 3 of the CSF samples had sufficient volume remaining for the measurement of specific thrombin activity. In these 3 samples, 34±7% of the total protease activity was inhibited by 50 U hirudin.

Effects of Repeated Administration of Human SAH CSF on Tension Generated by Cerebral Artery and Role of Thrombin
It was observed that the tension generated in canine verteobasilar arterial segments in response to application of a sample of SAH CSF was greater if the arterial segment had been exposed previously to that sample of CSF. Both the first and second administrations of CSF caused concentration-dependent contractions (Figure 2; first administration, F=3.8 and P<0.01; second administration, F=10.3 and P=0.001). However, tension generated by SAH CSF applied to a fresh cerebral artery was significantly less than the tension generated by the same CSF sample applied to a cerebral arterial segment that had previous been exposed to the CSF (F=15.7, P<0.001). A significant difference between the 2 groups was noted with as little as a 10⁻⁷ dilution of SAH CSF.

This observation led to the hypothesis that the initial exposure to SAH CSF altered the cerebral arterial responsiveness to some factor within the CSF in a manner that was not susceptible to washout and that was long-lived. Hemoglobin was eliminated as this factor because repeated application of purified oxyhemoglobin does not cause consecutively greater increases in tension (data not shown). Rather, the activity of a blood-borne proteolytic enzyme was suspected, particularly that of thrombin. To test the involvement of thrombin, its enzymatic activity in the CSF samples was blocked by pretreating the arterial segments with hirudin, a highly

\[
[methemoglobin] = \frac{[279-A_{577nm}] - (30-A_{577nm})}{4}
\]

Several random supernatant fluids from the monkey subarachnoid blood clot were subject to spectrophotometric analysis to determine the degree to which hemoglobin had been removed from the solution by trichloroacetic acid treatment (Figure 1). This was necessary because hemoglobin exhibits a pronounced absorption curve overlying the 405-nm wavelength (the Soret band) that would interfere with the measurement of free nitroanilide. Specific measurement of the 405-nm wavelength (the Soret band) that masks the nitroanilide peak at 405 nm. C, The same sample in panel B after trichloroacetic acid treatment and neutralization with sodium hydroxide.

Drugs and Proteins
Thrombin, plasmin, Cromozyme, antifoam B, inorganic ions, acids, and bases were purchased from Sigma Biochemicals. Thrombin, plasmin, and hirudin were purchased in lyophilized form and were dissolved in Krebs’ buffer immediately before use. Cromozyme was purchased in lyophilized form and was diluted with distilled water to a stock concentration of 1 mg/mL.

Hirudin was purchased from American Diagnostica Inc. Hirudin is a well-described inhibitor of thrombin (see References 16 and 19). There are reports that hirudin inhibits the enzymatic activity of plasmin17 and factor IX16; however, these have been contradicted (thrombin,16 factor IX30). Hirudin has been shown not to inhibit a variety of other proteases.21–26

Purified human hemoglobin was the generous gift of Hemosol Inc. Spectrophotometric assessment of the hemoglobin stock solution showed that it was purely in the oxyhemoglobin form. Purified
specific antagonist of thrombin. Figure 3 shows the effect of hirudin on contractions caused by SAH CSF samples on arteries with or without prior exposure to SAH CSF. Hirudin by itself had no effect on resting cerebral artery tension (prehirudin tension 0.64 ± 0.02 g, posthirudin tension 0.63 ± 0.02 g; t = 0.79, P = 0.22). However, hirudin pretreatment significantly reduced tension generated by the subsequent administration of SAH CSF onto fresh cerebral arterial segments (F = 13.5, P < 0.001). Significantly greater tension was generated in the presence of hirudin after administration of as little as 3.2 × 10⁻¹ dilution of CSF. In contrast, hirudin did not significantly affect the tension generated by SAH CSF on arterial segments that had been previously treated with the CSF (F = 1.4, P = 0.23).

Effects of Thrombin on Oxyhemoglobin Vasocostriction
Responses of arterial segments to purified thrombin were assessed. Thrombin caused concentration-dependent contractions (Figure 4, F = 4.5, P < 0.001) that could be antagonized by pretreatment with 5 U hirudin (F = 0.2, P = 0.97; data not shown). Thrombin in our experiment did not induce vasorelaxation; this is likely because of the lack of functional endothelium in our arterial segments, as was demonstrated by lack of relaxation in response to ATP (10⁻⁵ mol/L) administered after contracting the arterial segments with KCl (45 mmol/L) (data not shown).

Because oxyhemoglobin is believed to be an important vasoconstrictor in SAH CSF, the effects of thrombin, oxyhemoglobin, and thrombin plus oxyhemoglobin on tension generated by arterial segments were compared (Figure 4). The effect of thrombin is described above. Oxyhemoglobin administered alone caused concentration-dependent increases in tension (F = 3.9, P < 0.001). Combined administration of thrombin plus oxyhemoglobin also caused concentration-dependent increases tension (F = 12.5, P < 0.001), but the response was significantly different from that of either thrombin or oxyhemoglobin administered alone (F = 1.8 and P < 0.001 versus thrombin alone, F = 13.9 and P < 0.001 versus oxyhemoglobin alone). Tension generated by arterial segments exposed to oxyhemoglobin plus thrombin was less than that generated by oxyhemoglobin alone over thrombin concentrations from 10⁻⁴ to 10⁻² U/mL, whereas at the highest doses of thrombin and oxyhemoglobin, the contraction was greater than that generated by oxyhemoglobin alone.

Measurement of Thrombin Enzymatic Activity in Monkey Subarachnoid Blood Clot
The enzymatic activity of thrombin in monkey subarachnoid blood clots was measured over time to ascertain at what times...
after SAH thrombin might be acting to limit vasoconstriction. The assay demonstrated that the total protease activity in the clot decreased very rapidly during the first 3 days after placement of the blood clot into the subarachnoid space (Figure 5). Hirudin-sensitive chromogenic activity (eg, thrombin activity) accounted for about 46% of the total protease activity 1 day after placement of the clot. This result is similar to the fraction of thrombin activity in a fresh blood clot that was not placed in the subarachnoid space (48%), represented by the day-0 time point. Thrombin activity appears to decay more rapidly than does total protease activity, inasmuch as excess hirudin inhibited only 17% of the total protease activity in clots removed after 5 to 7 days. By 7 days after clot placement, this is equivalent to 0.002 U/mL thrombin activity in the clot.

**Effect of Thrombin on Tension Generated by Other Vasoconstrictors**

The specificity of the interaction between thrombin and oxyhemoglobin was examined by assessing the ability of thrombin to alter the contraction generated by KCl (60 mmol/L) and endothelin-1 (2 × 10^{-11} mol/L). Arterial segments were exposed to thrombin (0.32 U/mL), and after achievement of maximal contraction, either KCl or endothelin-1 was administered without washing out the thrombin. These contractions were compared with those caused by KCl or endothelin-1 in the absence of thrombin.

Figure 4. Effects of administering thrombin, oxyhemoglobin, and thrombin plus oxyhemoglobin on cerebral arterial tension ex vivo. Serial dilutions of thrombin (10 U/mL, solid circles, n=13 trials), oxyhemoglobin (10 g/dL, stippled squares, n=13 trials), or thrombin plus oxyhemoglobin (stippled triangles, n=9 trials) were sequentially administered onto canine vertebrobasilar arterial segments in tissue culture baths. Each solution or combination of solutions was administered onto a fresh arterial segment. Data are expressed as percentage of tension generated in response to 60 mmol/L KCl. *P<0.05 and #P<0.05 vs oxyhemoglobin (significant difference).

Figure 5. Time course of thrombin activity levels in a blood clot placed in the subarachnoid space. A Spectrophotometric assay was used to quantify the amount of total protease activity (solid circles) in the subarachnoid blood clot. A blood clot was surgically placed in the subarachnoid space of monkeys, and after a period of 1 day (n=3), 3 days (n=4), 5 days (n=4), or 7 days (n=4), it was removed, and its thrombin activity was measured. The zero time point represents a fresh blood clot that was not placed in the subarachnoid space (n=9). Specific thrombin activity (stippled squares) is defined as amount of total protease activity that was inhibited by excess hirudin (50 U).

Figure 6. Effects of thrombin pretreatment on contraction generated by KCl or endothelin-1. Thrombin (0.32 U/mL) was administered onto ex vivo arterial segments, allowing maximal contraction to develop. Without washing out the thrombin, either KCl (60 mmol/L, n=6 trials) or endothelin-1 (2 × 10^{-11} mol/L, n=6 trials) was then administered. The tension generated after administration of KCl or endothelin-1 was compared against that generated by these substances in the absence of thrombin (n=12 trials for KCl, n=12 trials for endothelin-1). Data are expressed as change in tension in milligrams generated in response to KCl or endothelin-1.

precontraction (Figure 6). Pretreatment with thrombin did not affect the magnitude of contraction to KCl (t=0.16, P=0.87) or endothelin-1 (t=0.94, P=0.37). Thus, it is unlikely that the action of thrombin in reducing the contraction caused by...
din significantly increased the tension generated by the first specific thrombin antagonist hirudin. Pretreatment with hirudin determined thrombin vasoactivity in SAH CSF by using the have been identified in cerebral arteries. Therefore, we present in the CSF in proportion to the severity of SAH.11 As a potential candidate for the relaxant factor. Thrombin is artery to CSF. Several observations have suggested thrombin as a general effect of thrombin on all vasoconstrictive substances.

**Effects of Plasmin on Oxyhemoglobin Vasocostriction**

To further study the nature of the interaction between oxyhemoglobin and thrombin, tension responses to oxyhemoglobin in the presence of plasmin were examined. Plasmin alone did not affect cerebral arterial tension (Figure 7; Figure 7). Furthermore, plasmin did not affect the contractile response to oxyhemoglobin. Rather, oxyhemoglobin coadministered with plasmin caused a dose-related increase in arterial tension (Figure 7; Figure 7). At the 0.32 and 1 U/mL doses) was greater than the response in the absence of plasmin (Figure 7; Figure 7). The aforementioned relationship between thrombin and oxyhemoglobin appears to be specific. The reduction of oxyhemoglobin vasoconstriction was selective for oxyhemoglobin because thrombin did not reduce the tension generated by other vasoconstrictor agents. Furthermore, the serine protease plasmin did not reduce oxyhemoglobin-induced contraction of cerebral arterial segments. In fact, high concentrations of plasmin potentiated oxyhemoglobin-induced contraction. Plasmin has been reported to enzymatically cleave protease-activated receptor-1, a thrombin receptor, and to cause desensitization of biological responses to thrombin. Because plasmin did not cause any contraction by itself in our hands (see Reference 8 for a contrary finding), we are left with the possibility that the augmentation of oxyhemoglobin contractility by plasmin is the result of an elimination of an endogenous thrombin vasorelaxing mechanism that is activated by high concentrations of oxyhemoglobin.

The observations in the present study appear to reflect the effects of oxyhemoglobin and thrombin directly on the smooth muscle of the cerebral artery. We used a preparation of cerebral artery that was deficient in endothelium so that thrombin would not be able to reduce artery tension via its well-described endothelium-dependent mechanism. Addi-
tionally, the absence of a functional endothelium more closely represents a vasospastic artery, which has been shown to have reduced endothelium-dependent vasorelaxant ability.31 Recent studies suggest that the influx of extracellular calcium is an important component in the contraction of smooth muscle caused by oxyhemoglobin.32 On the other hand, thrombin receptors within cerebral artery smooth muscle cells open membrane calcium channels to cause vasoconstriction.10 How such mechanisms might interact to account for the observations reported in the present study is unknown. Thrombin has been proposed as a causative agent in vasospasm because of its vasoconstrictive action on cerebral arteries and its presence in the subarachnoid clot.33–36 By extension, a protective role for thrombin antagonists against the development of vasospasm has been proposed.7 Part of the discrepancy between that hypothesis and the findings in the present study may be the vasoactivity of fibrinopeptides, which are the products of the cleavage of thrombin to fibrinogen. Fibrinopeptides are known to cause vasoconstriction in various peripheral arteries.37 Also, high levels of the breakdown products of fibrin have been associated with vasospasm.38 In fact, evidence has been presented that shows that thrombin facilitates the development of vasospasm only through the production of fibrinopeptides. Arutunov et al19 demonstrated severe vasoconstriction of canine basilar artery after placement of a fibrinogen-thrombin mix around the artery, but no vasoconstriction was observed if either substance was placed around the artery alone. Thus, the association of thrombin with the development of vasospasm may be a spurious one, and instead, we propose that the products of thrombin enzymatic activity promote vasospasm rather than thrombin per se.

In summary, we propose that thrombin reduces the contraction of cerebral arteries caused by hemoglobin and that this occurs within SAH CSF. This interaction between thrombin and oxyhemoglobin appears to be independent of the endothelium and is specific for the 2 substances. On the basis of the time course of thrombin activity in the subarachnoid clot, we hypothesize that the ability of thrombin to reduce hemoglobin contractility accounts in part for the delayed onset of vasospasm until usually 3 or 4 days after SAH. Furthermore, the potency of this action of thrombin allows for the possibility that thrombin limits the contraction generated by SAH CSF even after vasospasm has developed.

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