Vasodilator Proteins: Role in Delayed Cerebral Vasospasm

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SUMMARY Blood proteins could play a critical role in the pathogenesis of cerebral vasospasm in subarachnoid hemorrhage (SAH) as agonists and as antagonists of vasoconstriction. The present study was designed primarily to quantify the inhibition produced by antithrombin III of the phasic responses elicited by cumulative doses of KCl, serotonin (5-HT), and thrombin in isolated canine cerebral arteries. Kallikrein (1 and 4 units/ml) did not inhibit UTP but inhibited high K+ and 5-HT through an effect on the endothelium. Kallikrein (1 unit/ml) irreversibly blocked the responses to thrombin. Globulins (0.1 mg/ml and 0.4 mg/ml) inhibited the contractile responses to high K+, 5-HT, and thrombin. Kaiklikrein (1 and 4 units/ml) did not inhibit UTP but inhibited high K+ and 5-HT through an effect on the endothelium. Kaiklikrein (1 unit/ml) irreversibly blocked the responses to thrombin. Globulins (0.3 mg/ml) and fibrinogen (0.5 mg/ml) were not inhibitory.

The results demonstrate that anticoagulant proteins are very effective nonspecific inhibitors of the vasoconstriction, whereas the serine protease kaiklikrein selectively blocks thrombin. The remarkable potency of antithrombin III suggests that it may protect cerebral arteries from exhibiting vasospasm in SAH.

A COMMON FEATURE OF SUBARACHNOID HEMORRHAGE (SAH) is the occurrence of cerebral vasospasm. The incidence of severe vasospasm in SAH is related to the volume of intracranial blood, and the manifestations of brain ischemia in turn are related to the magnitude of the vasospasm. One characteristic of cerebral vasospasm that has eluded an explanation is the delayed nature of the phenomenon. The median time for its occurrence is about 7 days after...
and effective vasorelaxant, so that its disappearance from the cerebrospinal fluid (CSF) after hemorrhage may be a requisite for the occurrence of cerebral vasospasm.11 Because of the possibility that antithrombin III might naturally protect cerebral arteries from vasospasm in cases of SAH, the present study was performed to better define and quantify the vasorelaxant properties of this anticoagulant and to ascertain whether another anticoagulant, alpha2-macroglobulin, might produce a similar effect on isolated cerebral arteries. Kallikrein has been included in this study because, in contrast to other serine proteases previously tested, it failed to elicit a contractile response in cerebral arteries. Instead, it was found to inhibit the contraction generated by some agonists, especially the vasoconstriction obtained with thrombin.

Material and Methods

Mongrel dogs (16-20 kg) of either sex were anesthetized with pentobarbital sodium (30 mg/kg) and exsanguinated. The brain was removed and the outside and lumen of the basilar artery were washed in situ with a physiological salt solution (PSS). The basilar artery was then dissected free, placed in a Petri dish containing PSS, irrigated again, and a 4 mm segment was isolated for study. This segment was slotted onto two parallel prongs of a tissue chamber.13 The chamber was filled with 10 ml of PSS having the following composition (mM): NaCl, 118.3, KCl, 4.7, MgSO4, 1.2, NaHCO3, 25, KH2PO4, 1.2, CaCl2, 2.5 and glucose 11.0. The pH was adjusted to 7.4 by adding HCl. A mixture of 95% O2 and 5% CO2 aerated the tissue bath mixture of 95% O2 and 5% CO2 aerated the tissue bath. The pH of the aerated bath was 7.34 at 39°C.

Two segments of the artery were studied on the same day in two separate tissue chambers. The unused portion of the vessel was stored refrigerated and sectioned the following day for study. The type of experiment performed with each of the segments differed, so that any peculiarity of one basilar artery did not dominate the overall findings.

The arterial segment was allowed to incubate for 1.5 hours, during which time it was washed with PSS every 20 minutes. This wash was an irrigation from the bottom of the tissue chamber to an overflow so that the arterial ring was not exposed to air during wash out. Arterial tension was initially set at 2 g by means of a fine positioning device (FTA 1001, Hewlett-Packard). Later, the responsiveness and the stability of the response for each artery was tested 2 or 3 times with KCl (10, 30 and 50 mM). Papaverine (3 x 10^-6M) was used to produce complete relaxation and to assess the amount of myogenic tone present in each arterial segment. Drugs were washed out with 180 ml of the buffer solution and 20 minutes elapsed between trials.

The isometric force generated during contraction was recorded by means of a Statham strain gauge transducer (11388, GL-1.5–300) and a Grass Model 7 polygraph. Calibration was performed by using standard gram weights. The isometric force recorded was expressed in grams. The responses were computed as mean ± S.E.M., and significant differences between these values were determined by the Student's t-test for unmatched pairs. Probabilities of less than 5% (p < 0.05) between control and experimental values were considered to be significant.

The proteins and drugs used in this study were human antithrombin III, human alpha2-macroglobulin, porcine kallikrein, human alpha, beta, and gamma globulins, human fibrinogen, human thrombin, uridine triphosphate (UTP), serotonin creatinine sulfate (5-HT), ouabain, and papaverine (Sigma Chem. Co., St. Louis). The proteins were dissolved in 0.9% NaCl, or in PSS, and the drugs in water. The volume of solutions added to the bath in most cases was 10 μl to 100 μl and did not exceed 3% of the bath solution. However, when the globulins and fibrinogen were used, 1 ml of the bath solution was removed prior to adding 1 ml of warmed PSS containing these proteins.

Preliminary experiments showed that alpha2-macroglobulin and kallikrein would not induce a contraction when applied to the basilar artery but would inhibit arteries precontracted with KCl or 5-HT. In this regard, these proteins resembled antithrombin III,11 inhibiting the tonic phase of the contraction produced by agonists. However, in most of the present experiments, the inhibitory proteins were administered prior to the contractile agonists primarily to determine their effect on the phasic portion of contraction. The rate of decay of the tonic phase varies greatly with the type of agonist used, as well as with the preparation, making it more difficult to quantify inhibition.11

The inhibitory effect of the proteins was quantified by adding the protein to the bath 2 min prior to the application of cumulative doses of contractile agonists. The highest concentration of KCl (5 x 10^-3M), 5-HT (10^-4M), UTP (10^-4M) and thrombin (1 unit/ml) used in these experiments produced maximal contraction of the cerebral artery (Cmax). Control dose-response curves for thrombin-induced contractions were obtained in different arteries (n = 10) from those used to test for inhibition, because thrombin produces tachyphylaxis in the canine basilar artery.11 In most experiments, two concentrations of antithrombin III (1 and 3 units/ml), alpha2-macroglobulin (0.1 and 0.4 mg/ml) and kallikrein (1 and 4 units/ml) were tested in the same vessel for inhibition. Except in the case of thrombin, control dose-response curves for the contractile agonist were obtained before the application of the lower concentration of the inhibitory protein and occasionally prior to the application of the higher concentration, so that at least 10 control responses were used for statistical analysis. In some experiments the same dose of the inhibitory protein was tested a second time to see if an identical result was obtainable. Recovery of the artery was verified after wash out of the protein by applying KCl and, except in the case of thrombin, by applying the other contractile agonist under study. Further details are presented in Results.

Some experiments were performed in basilar arteries that had been reamed to destroy the endothelium14
in order to see if this procedure prevented vasorelaxation. Methacholine (10^{-5} M) will relax precontracted cerebral arteries (UTP, 10^{-5} M) only when the endothelium is intact and this procedure was used to confirm that reaming had removed the endothelium. Three experiments were performed on 4 mm rings of the mesenteric artery to determine if antithrombin III might relax a peripheral artery.

### Results

#### Effects of Antithrombin III

Figure 1 summarizes the inhibitory effects of antithrombin III on the dose-response curves produced by a variety of contractile agonists. In all cases 1 unit/ml of antithrombin III significantly inhibited the maximal contractions produced by high K^+ (tracing A) and 5-HT (tracing B). In two experiments (not shown) 3 units/ml antithrombin III was applied three times without producing tachyphylaxis to the inhibition.

In order to ascertain whether the endothelium was essential for the vasorelaxant property of antithrombin III, experiments were performed on reamed basilar arteries (fig. 3). These arteries were precontracted with either high K^+ (n = 3) or ouabain (n = 2). In either case, antithrombin III relaxed the vessel in a persistent manner (fig. 3).

Three experiments were performed on nonreamed segments of mesenteric arteries to see if antithrombin III would relax a peripheral vessel precontracted with high K^+ (80 mM). The persistent vasorelaxant effect obtained with antithrombin III in these vessels is illustrated in figure 3.

#### Effects of Alpha2-macroglobulin

The general pattern of inhibition produced by alpha2-macroglobulin is summarized in figure 4. In 0.1 mg/ml concentration, alpha2-macroglobulin significantly reduced C_max elicited by high K^+ (tracing A), 5-HT and thrombin. The contractions produced by the middle concentration of these agonists were also significantly inhibited, as were the responses produced by the lowest doses of 5-HT and thrombin. The effect of thrombin was clearly more affected by 0.1 mg/ml alpha2-macroglobulin than were the effects of high K^+ and 5-HT. The thrombin-induced contractions were, how-

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**FIGURE 1.** Effects of 1 and 3 units/ml of antithrombin III (AT) on dose-response curves of KCl, 5-HT, UTP, and thrombin. Numbers in parentheses are number of experiments.

**FIGURE 2.** Tracings of the inhibitory effects of antithrombin III (AT) and alpha2-macroglobulin (A2-M) on contractions produced by cumulative doses of K^+ or 5-HT. The proteins were added prior (arrows) to the contractile agonists. Doses of proteins represent concentrations per ml.
ever, as much inhibited by 0.1 mg/ml of alpha₂-macroglobulin as by the 0.4 mg dose (fig. 4). In contrast, 0.4 mg/ml of alpha₂-macroglobulin significantly depressed the middle and high dose responses of K⁺ and 5-HT more than the 0.1 mg dose (fig. 4). A tracing showing the inhibitory effect of 0.4 mg/ml alpha₂-macroglobulin on the contractions elicited by 5-HT is shown in figure 2, tracing C.

The C₉₀ produced by high K⁺ in these experiments was 7.87 ± 0.39 g. The maximal effect induced by 5-HT was 10.11 ± 0.6 g and by thrombin 7.65 ± 0.68 g.

In two experiments the basilar artery was exposed to 0.4 mg/ml of alpha₂-macroglobulin for 1 hr before high K⁺ was applied. The maximal response to K⁺ was inhibited about 34%, suggesting that the artery had not become refractory to the inhibitory effect during the hour interval.

**The Effects of Kallikrein**

As shown in figure 5, a small dose of kallikrein (0.1 unit/ml) markedly inhibited and 1.0 unit/mg abolished the contractile effect of thrombin. Moreover, when the artery was exposed to 1.0 unit/ml kallikrein for 30 min, washed out, and 20 min later exposed to thrombin no response was seen (n = 4). This result indicates that the inhibition to thrombin is irreversible. Figure 6 illustrates the finding that the contraction produced by thrombin was much more affected by low doses of kallikrein than was the case with 5-HT.

Kallikrein in either 1 or 4 units/ml doses significantly inhibited the contractions elicited by high K⁺ or 5-HT (fig. 5). However, the inhibition produced by the 4 unit dose was not statistically greater than that obtained with 1 unit/ml kallikrein. In contrast, either dose of kallikrein given 2 min before UTP failed to inhibit the contractile response (fig. 5).

The inhibitory effect of kallikrein to the responses produced by high K⁺ was apparently due to an effect on the endothelium, because 4 units/ml kallikrein failed to inhibit K⁺ contractions in reamed arteries (n = 3). The contractions elicited by 10, 30 and 50 mM K⁺ before and 2 min after the application of kallikrein (1 unit/ml) averaged 0.4, 5.8, 6.8 g and 0.2, 5.9, 7.1 g, respectively. Also, the inhibitory factor released from the endothelium was relatively short-lived because when the artery was exposed to 4 units/ml kallikrein for 30 min without wash out, the response to high K⁺ was not inhibited (fig. 5).

Reaming the artery also prevented kallikrein (4 units/ml) from altering the response to 5-HT (n = 2). In contrast, kallikrein (1 unit/ml) completely blocked the constrictor effect of thrombin (1 unit/ml) in reamed vessels (n = 2).

The C₉₀ for the agonists shown in figure 5 was 7.09 ± 0.35 g (left graph) and 7.66 ± 0.41 g for high K⁺, 8.49 ± 0.93 g for 5-HT, 7.65 ± 0.68 g for thrombin and 8.51 ± 0.78 g for UTP.

**Results with Globulins and Fibrinogen**

The concentrations of the globulins (3.0 mg/ml) and fibrinogen (0.3 mg/ml) used in these experiments was one-tenth of that present in plasma. This concentration was sufficient to serve as a suitable control for the vasoactive proteins (see Discussion). Higher concentrations produced an inordinate foam when added to the aerated bath. Because even low concentrations of albumin produced foam, this protein was not studied.

The globulins of Cohen fraction IV (alpha and beta-globulins) (n = 3) or gamma-globulin (n = 2) had no effect on the contraction produced by high K⁺. For instance the C₉₀ for K⁺ alone was 7.1 ± 0.97 g, and 2 min after the addition of the globulins (n = 5) this response was 7.2 ± 0.76 g. Likewise, fibrinogen had no effect (n = 2). The C₉₀ for high K⁺ before fibrinogen averaged 8.2 g and after fibrinogen 8.6 g. Also,
neither gamma-globulin (n = 2) nor fibrinogen (n = 1) altered the response to 5-HT.

Discussion

These results clearly demonstrate that the anticoagulants antithrombin III and alpha,-macroglobulin are effective inhibitors of cerebral arterial vasoconstriction. The results suggest that these proteins could protect the cerebral arteries from vasospasm following SAH. If so, the inhibition may account for the fact that many SAH patients do not manifest vasospasm, even those at high risk. Also, vasospasm typically occurs about one week following hemorrhage and this may depend in part upon the disappearance of disoparator proteins. Although there was no evidence of tachyphylaxis to the inhibitory effect of the anticoagulants, the inhibition was readily removed by wash out. This suggests that the normal washing action of the CSF would eventually terminate the inhibition.

It has been reported that 1 ml of plasma can neutralize 700 units of thrombin and that 70% to more than 90% of this antithrombin activity is attributable to antithrombin III. The concentrations of the anticoagulants used in this study were far less than the concentrations present in plasma, which contains about 0.3 mg/ml of antithrombin III and about 3.0 mg/ml of alpha,-macroglobulin. Thus the 1 unit/ml of antithrombin III used in these experiments represent approximately 1/490 to 1/630 of the antithrombin activity normally present in human plasma. Moreover, 1 unit of antithrombin III represents about 2 /ug/ml of protein in these experiments. The antithrombin activity of plasma due to alpha,-macroglobulin has been estimated to be 5% to 30% of the total and the 0.1 mg dose of this protein would be 1/30 of that found in plasma. Because of its larger size, alpha,-macroglobulin (MW = 820,000) might disappear from the CSF more slowly after SAH than antithrombin III (MW = 65,000), but the present results suggest that antithrombin III would play the most important role as an inhibitor of vasoconstriction in SAH. In contrast to some polypeptide vasodilators like VIP, antithrombin III did not induce tachyphylaxis. Antithrombin III is a remarkably stable and potent protein that inhibits a wide variety of contractile agonists, including plasmin and prostaglandin F2.

The finding that 10-5M ouabain, which vasoconstricts via Na+-K+-ATPase inhibition, failed to block the inhibitory effect of antithrombin III indicates that this protein does not relax the artery by stimulating the electrogenic sodium pump. The inhibitory effect was also independent of an action on the endothelium (fig. 3). This last finding suggests that, unless it leaked into the vessel wall, circulating antithrombin III would play no physiological role as a vasodilator. In contrast to some polypeptide vasodilators like VIP, antithrombin III did not induce tachyphylaxis. Antithrombin III is a remarkably stable and potent protein that inhibits a wide variety of contractile agonists, including plasmin and prostaglandin F2.

Such actions might delay the appearance of vasospasm which occurs late in the course of SAH.

The absence of a contractile response to kallikrein

units/ml of thrombin reported to be generated by plasma.

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The absence of a contractile response to kallikrein
was unexpected, because other serine proteases (trypsin, plasmin, and thrombin) elicit vasoconstriction in isolated canine basilar arteries. The results, however, suggest that kallikrein may act on the same receptor as thrombin. One unit/ml of kallikrein was sufficient to completely block the contraction to thrombin. In contrast, 4 units/ml of kallikrein failed to affect the response to UTP and, on average, reduced the maximal response to high K+ or 5-HT by less than 40% (fig. 5). Moreover, arteries that were exposed to 1 unit/ml kallikrein for 30 min and washed failed to respond later only to thrombin. Trypsin has a similar effect, in that arteries previously exposed to trypsin are subsequently refractory to thrombin. It is possible, therefore, that the irreversible inhibition obtained with kallikrein was due to a proteolytic action on the vessel wall that is common to serine proteases. Why kallikrein alone did not produce a contraction as the other proteases do requires further investigation.

In contrast to thrombin, the inhibition by kallikrein to the contractions generated by high K+ and 5-HT depended on a functional endothelium. The relatively weak and transient inhibition exerted by kallikrein on the contractions of K+ and 5-HT, and the absence of an effect on UTP, suggests that kallikrein would not protect arteries from manifesting vasospasm.

The first proteins thought to be involved in the pathology of SAH were hemoglobin (especially oxyHb) and thrombin. Both of these produce vasoconstriction. Other proteins reported to elicit vasoconstriction of cerebral arteries are membrane proteins of erythrocytes, FDP, trypsin, and plasmin. The fact that oxyHb and thrombin induce, respectively, little or no vasoconstriction in dog mesenteric arteries illustrates the importance of using cerebral arteries in studies designed to elucidate factors responsible for cerebral vasospasm. Moreover, antithrombin III and other protease inhibitors could chemically neutralize such proteases in the CSF after SAH. If not, the fact that thrombin and plasmin cleave fibronectin and laminin present in the basement membrane could have far-reaching effects on the nature of vasospasm. The CSF concentration of trypsin and other proteases, as well as peptidases, may increase with disease. Little is known of their effects, or their end products, on cerebral arteries. Also, thrombin is the most potent fibroblast mitogen and, therefore, might be responsible for the meningeal fibrosis and hydrocephalus that follows SAH. OxyHb is believed to be of paramount importance in the generation of free radicals and prostaglandins in SAH. Hence, vasoconstriction may be only one effect of proteins that influences the course of SAH.

On the other hand, antithrombin III and alpha-1-macroglobulin by inhibiting many proteases and by inhibiting vasoconstriction may delay the deleterious consequences of SAH. Another abundant blood protein, haptoglobin, may play a similar role. It inhibits the contractile response to oxyHb and it is also a potent inhibitor of prostaglandin synthesis. Haptoglobin has been given intrathecally to patients as a treatment for vasospasm with equivocal results. The remarkable potency and nonspecific inhibitory effect of antithrombin III suggests that given intrathecally it might be more efficacious than haptoglobin as a therapeutic agent.

Although no in vitro study can imitate the complex changes in milieu that accompany SAH, the evidence available clearly indicates that blood proteins could play critical roles in the pathogenesis of vasospasm. The present findings suggest that early in the course of SAH antithrombin III and alpha-1-macroglobulin would afford protection against vasospasm. If so, the occurrence of vasospasm would depend on their egress from the CSF.

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References

The Effect of Nicardipine on Neuronal Function Following Ischemia

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SUMMARY In cerebral ischemia, it has been proposed that calcium influx into neurons results in irreversible cellular injury during reperfusion.

We administered nicardipine, a dihydropyridine calcium entry blocker, by continuous subcutaneous infusion to twenty-five rats beginning before (PR) or following (PO) ischemia, and compared somatosensory evoked potentials (SEPs) to twenty-eight ischemic control animals. Comparable ischemic cellular changes were seen in the hippocampi of all animals.

SEP amplitude was higher in both the PR (p < .005) and PO (p < .0005) groups compared to controls. This effect was found in all three components (P₁, N₁, P₂) of the evoked response. Plasma nicardipine levels of 6–10 ng/ml were associated with mild hypotension.

We conclude that nicardipine improved neuronal function as measured by SEPs when administered before or after ischemia, most likely by interrupting the cytotoxic events occurring in cortical neurons during reperfusion.

THE PURPOSE OF THIS STUDY was to assess the effect of nicardipine, a dihydropyridine calcium entry blocker, on neuronal function in acute forebrain ischemia in rats. The drug was begun either prior to or immediately following ischemia and in both cases continued for 72 hours after the insult. The effect of this therapy was determined by measurement of cortical evoked potentials in treated and control animals.

Calcium blockers may be beneficial in cerebral ischemia because of two possible mechanisms: 1) relaxation of vascular smooth muscle and increase of cerebral blood flow (CBF), and 2) preservation of mitochondrial function in neurons by preventing the accumulation of intracellular calcium. It has been postulated that ischemia followed by reperfusion augments entry of this ion into neurons, and that calcium subsequently activates phospholipases producing a chain of events in the presence of oxygen resulting in the formation of damaging free radicals.

Pulsinelli’s four vessel ischemia model is appropriate for the evaluation of the second mechanism. Severe delayed ischemic neuronal damage is produced in regions of brain which are immediately reperfused, CBF returning to normal by 24 hours so that a cytotoxic chain of events must be precipitated during the initial period of ischemia. Somatosensory evoked potentials (SEP) have been...
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