Effect of intracisternal antithrombin III on subarachnoid hemorrhage-induced arterial narrowing

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The ability of antithrombin III, an endogenous plasma glycoprotein, to reverse the arterial narrowing in a rabbit model of cerebral vasospasm was evaluated. The vasodilator activity of antithrombin III on rabbit arteries was first assessed in vitro using a myograph-arterial ring preparation. Antithrombin III (10 IU/ml) induced a 55.4% ± 2.66% (mean ± standard error of the mean) relaxation in basilar artery precontracted with serotonin (5-HT) in five specimens as compared with a 9.8% ± 1.6% relaxation of common carotid artery in six specimens. For in vivo analysis, 21 New Zealand White male rabbits were separated into three groups: Group 1 served as normal controls; Group 2 received a subarachnoid blood injection (SAH) and were sacrificed on Day 3 thereafter; and Group 3 animals were subjected to SAH, then received a 2-hour intracisternal infusion of antithrombin III (100 IU) in saline prior to sacrifice on Day 3. Basilar artery caliber was determined using a morphometric method to analyze perfusion-fixed arterial segments. Control basilar artery diameter in Group 1 was 0.64 ± 0.02 mm. In Group 2 a 27% reduction in arterial caliber to 0.47 ± 0.03 mm was observed by Day 3 post SAH (p < 0.0001). Group 3 animals had a mean basilar artery diameter of 0.68 ± 0.02 mm. This was significantly larger than the untreated SAH rabbits in Group 2 (p < 0.0001), but not different from control artery diameters in Group 1. The findings demonstrate that antithrombin III in saline has a significant ability to reverse delayed narrowing of the rabbit basilar artery after SAH.

KEY WORDS • antithrombin III • cerebral artery • vasospasm • subarachnoid hemorrhage • rabbit

Cerebral vasospasm poses significant risks of cerebral ischemia and infarction to patients suffering aneurysmal subarachnoid hemorrhage (SAH). This arterial narrowing is characteristically delayed in onset and in proportion to the amount of blood deposited in the subarachnoid space. Numerous substances have been implicated in the etiology of this phenomenon; however, extensive research has not determined the predominant pathophysiology. Some researchers have emphasized the importance of vasculopathic changes in producing arterial narrowing, while others have focused on the roles of denervation hypersensitivity or vasoconstrictors deposited in the periarterial milieu. The relative importance of these varied pathogenetic mechanisms in the etiology of vasospasm has yet to be defined. Nonetheless, it is likely that an element of vasoconstriction is involved based on an analysis of wall mechanics and laboratory and clinical observations of partial or transient reversal of vasospasm by vasodilator substances. The development of a safe pharmacological agent which could satisfactorily reverse the delayed arterial narrowing that accompanies SAH would have great therapeutic potential.

Recently, antithrombin III, an endogenous plasma glycoprotein that inhibits a number of serine proteases, has been shown to produce endothelium-independent vasodilatation in vitro. The vasodilatation in response to application of this agent did not demonstrate tachyphylaxis, nor was this vasorelaxant effect specific to any certain constrictor agonists. These potent cerebral vasodilator effects suggested a potential therapeutic role for antithrombin III in the treatment of cerebral vasospasm. The present experiments were designed to assess the effectiveness of antithrombin III as a vasodilator of the rabbit basilar artery in vitro, using isolated ring segments, and in vivo in a rabbit model of delayed vasospasm following SAH.

Materials and Methods

In Vitro Experiments

To assay the vasodilator activity of antithrombin III, in vitro experiments using isolated ring segments of rabbit basilar artery and rabbit common carotid artery
were performed. Adult male New Zealand White rabbits, each weighing 2.9 to 3.4 kg, were anesthetized with an intramuscular injection of a mixture of ketamine (20 mg/kg), xylazine (5 mg/kg), and acepromazine (0.25 mg/kg) and were sacrificed by exsanguination from the femoral artery. The brain with the basilar artery in situ was removed and placed in a dissecting chamber filled with a modified Krebs bicarbonate solution with a composition of (in mM): NaCl 120, KCl 4.5, MgSO\(_4\) 1.0, NaHCO\(_3\) 27.0, KH\(_2\)PO\(_4\) 1.0, CaCl\(_2\) 2.5, and dextrose 10.0. The basilar artery was dissected free under magnification and divided into 3-mm segments. The ring segments were suspended between two L-shaped stainless steel rods in an organ bath with a 10-ml working volume of Krebs solution, which was gassed with 95% O\(_2\) and 5% CO\(_2\). Ring segments of the cervical common carotid artery were also prepared and suspended in identical organ baths. The pH of the bathing solution ranged from 7.4 to 7.5. One rod was connected to a force-displacement transducer.\*

The preparations were allowed to equilibrate at 37°C for 60 minutes prior to use. Resting tension was adjusted to 400 mg for basilar artery segments and 3.0 gm for common carotid artery segments. Contractile force was recorded isometrically using a force-displacement transducer and was displayed on a polygraph.† To confirm sufficient contractile activity in each specimen, the response to 40 mM KCl was first obtained in each ring segment, and only specimens which showed a good response were used for subsequent experiments.

The response to incubation with 40 mM KCl and 10\(^{-6}\) and 10\(^{-5}\) M serotonin (5-HT) was measured. Arteries precontracted with KCl or 5-HT were then treated with graded concentrations of antithrombin III (1 IU/ml, 3 IU/ml, and 10 IU/ml) and the response was recorded as a percent reduction of the initial agonist-induced contraction.

In Vivo Experiments

Twenty-one male New Zealand White rabbits, each weighing between 3 and 4 kg, were randomly separated into three experimental groups. Group 1 animals served as a control (normal) group; Group 2 animals received a subarachnoid blood injection (SAH) and were sacrificed on Day 3 post-hemorrhage; and Group 3 animals were subjected to SAH, then given a 2-hour intracisternal antithrombin III (Group 3) were anesthetized, and the dura overlying the cisterna magna was operatively exposed. A No. 24 Teflon catheter was inserted into the cisterna magna under direct vision and secured in place. An infusion/withdrawal pump was used to deliver continuous infusion of antithrombin III at a rate of 12 \(\mu\)l/min for 2 hours (total volume infusion 1.44 ml). One hundred units of antithrombin III was dissolved in the volume infused.

During the infusion of antithrombin III, arterial pressure was continuously monitored and arterial blood gas tensions were periodically obtained. Controlled venti-

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\* Force-displacement transducer manufactured by Grass Instruments Co., Quincy, Massachusetts.

† Polygraph, Model 3418, manufactured by Soltex Corp., San Fernando, California.

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<table>
<thead>
<tr>
<th>Animal Group</th>
<th>No. of Animals</th>
<th>Body Weight (kg)</th>
<th>MAP</th>
<th>pCO(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: control</td>
<td>7</td>
<td>3.30 ± 0.07</td>
<td>76.4 ± 5.5</td>
<td>38.3 ± 1.55</td>
</tr>
<tr>
<td>2: SAH</td>
<td>7</td>
<td>3.20 ± 0.19</td>
<td>70.3 ± 2.8</td>
<td>38.5 ± 1.47</td>
</tr>
<tr>
<td>3: SAH + AT III</td>
<td>7</td>
<td>3.68 ± 0.18</td>
<td>77.1 ± 4.2</td>
<td>36.4 ± 4.02</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means. SAH = subarachnoid hemorrhage; AT III = antithrombin III; MAP = mean arterial pressure.
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ulation was adjusted to maintain the arterial \( \text{pCO}_2 \) between 35 and 45 mm Hg. Supplemental oxygen was introduced into the inspired air to maintain the \( \text{pO}_2 \) greater than 100 mm Hg. Anesthesia was supplemented as needed with intravenous ketamine (1 to 4 mg/kg/30 minutes).

**Perfusion Fixation and Tissue Preparation**

At the termination of the experiment, animals from all groups were perfusion-fixed in an identical manner. Anesthesia was supplemented, the thorax was opened, and a cannula was placed in the left ventricle. Immediately prior to perfusion, the right atrial appendage was opened and the descending thoracic aorta was clamped. The vascular system was flushed with 300 ml of Hanks' balanced salt solution (HBSS), pH 7.4, at room temperature, followed by 500 ml of 1% paraformaldehyde, 1.5% glutaraldehyde in HBSS (pH 7.4). Perfusion was performed at 75 mm Hg. After perfusion fixation, the brain was removed and immersed in fixative. The presence of a subarachnoid clot was visually confirmed. The brains were stored at 4°C overnight.

Basilar artery segments 2 mm in length were then cut from the vessel 2 mm above the vertebral confluence. The specimens were washed several times in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). The tissue was dehydrated in graded alcohol and embedded in Epon 812. Sections 1 μ thick were cut on an ultramicrotome, mounted on glass slides, and stained with toluidine blue for light microscopy. Photomicrographs at standard magnifications were made of a stage micrometer and then of at least five random arterial sections per specimen. The photographs were enlarged to the maximum size that could be printed on 8 x 10-in. photographic paper. Morphometric measurements were made using an image analyzer.* Three sets of measurements were made per section, for a total of 15 sets of measurements per specimen. The circumference of the lumen and the luminal area were directly measured. Arterial diameter was then calculated from these measured values.

**Drugs and Solutions**

Serotonin was dissolved in 0.1 N KCl with 0.1% ascorbic acid to make stock solutions; the KCl was dissolved in distilled water. For *in vitro* experiments, each drug was then dissolved in Krebs solution before use; less than 0.1 ml was added to the organ bath.

Human antithrombin III was provided in a lyophilized form.† The agent was reconstituted with distilled water for *in vitro* experiments and with sterile saline for cisternal infusions. Concentrations of antithrombin III are expressed in international units per ml (IU/ml). The pH of antithrombin III in saline is 6.8.

* Kontron Videoplan image analyzer manufactured by Carl Zeiss Co., Oberkochen, West Germany.
† Antithrombin III kindly donated by Hoechst-Roussel, Somerville, New Jersey.

Fig. 1. Relaxation responses of rabbit common carotid and basilar arteries *in vitro* to incubation with graded concentrations of antithrombin III (AT III). Arteries were precontracted with 40 mM KCl (left) and with serotonin (5-HT: 10⁻⁵ M for basilar artery and 10⁻⁶ M for common carotid artery, right).

**Statistical Methods**

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using a general linear model procedure for analysis of variance. Significance was assumed for comparisons where \( p < 0.05 \).

**Results**

**In Vitro Experiments**

Following KCl-induced precontraction, antithrombin III produced minimal relaxation in rabbit common carotid artery (4.6% ± 1.4%) even at the relatively high concentration of 10 IU/ml. Basilar artery, on the other hand, exhibited a 26.3% ± 3.3% relaxation when antithrombin III at a concentration of 10 IU/ml was added to arteries precontracted with 40 mM KCl (Fig. 1 left). Antithrombin III demonstrated greater potency in relaxing arteries precontracted with 5-HT (Fig. 1 right). Basilar artery relaxed 55.4% ± 8.6% of 5-HT-induced contraction in response to 10 IU/ml of antithrombin III. The effects of antithrombin III on common carotid artery were less potent, with only a 9.8% ± 1.9% relaxation for vessels treated with 10 IU/ml of antithrombin III.

**In Vivo Experiments**

During intracisternal blood injection most animals demonstrated transient arterial hypertension and pupillary nonreactivity which resolved within a few minutes. The animals were noted to be initially apathetic following SAH but demonstrated no focal neurological deficits and exhibited normal food and water intake.

Clinical parameters noted at the time of perfusion fixation are listed in Table 1. No significant differences were observed in mean weight or arterial blood gas values or MAP between experimental groups. No hemodynamic changes were noted during or following intracisternal infusion of antithrombin III. Subarach-
Discussion

The results of the present studies indicate that antithrombin III acts to dilate rabbit basilar artery in vivo in a manner similar to that previously observed with canine and human basilar arteries. While the qualitative response of the vessels to antithrombin III exposure was the same, some differences in sensitivity to antithrombin III were noted. In our experiments, relaxations in response to antithrombin III concentrations of less than 10 IU/ml were generally under 25%. At the highest concentration utilized (10 IU/ml), the maximum relaxation observed was 55.4% ± 8.6%. As reported by White, antithrombin III was more effective in relaxing 5-HT-induced contractions than those induced by KCl. These differences in effectiveness, however, were much more marked in our series than in those previously reported. Several explanations for these differences are possible, including species differences in sensitivity to antithrombin III or differences in the biological activity of the antithrombin III preparations used by us and White. In addition, methodological variation between the studies may in part account for the differences seen. The purpose of our present investigation was to establish that antithrombin III had cerebral vasorelaxant effects in the rabbit and to determine its potency in this regard prior to performing in vivo studies. Based on these studies a dose of antithrombin III was selected for our in vivo experiments which would assure a CSF concentration above 10 IU/ml during the time of infusion. To achieve this, 100 IU of antithrombin III was infused over 2 hours.

Antithrombin III produced a complete reversal of the arterial narrowing usually seen 3 days following the subarachnoid injection of blood. No morphological changes were observed at the light microscopic level in terms of intimal or medial proliferative changes or disruption of the internal elastic lamina. While careful morphometric analysis and ultrastructural study were not performed as part of the present investigation, previous morphometric work in our laboratory has established that the arterial narrowing seen in the rabbit model is not associated with gross structural changes and is therefore more likely to be vasoconstrictor in etiology. Previous experiments in our laboratory using an identical protocol have also shown that a 2-hour intracisternal saline infusion alone produced a statistically significant increase in basilar artery diameter on Day 3 after SAH (mean basilar artery diameter ± SEM (mm): control group 0.64 ± 0.02; SAH group 0.47 ± 0.03; SAH plus saline infusion group 0.60 ± 0.06). Infusion of saline, however, unlike the saline and antithrombin III infusions in the present study, did not return arteries to control diameters. Thus, although a portion of the present findings may be attributed to “washing away” perivascular spasmogens or to another action of saline infusion, antithrombin III also exerted a dilator influence.

Little information is presently available as to the possible mechanisms by which antithrombin III may be producing reversal of this delayed arterial spasm. The in vitro results obtained in our study as well as those by White and Robertson suggest that antithrombin III can be a potent vasodilator in cerebral arteries precontracted with a number of agonists. Thus, it is probable that antithrombin III is producing direct effects at the level of the vascular smooth muscle. It is possible that antithrombin III has its effect on vasospasm by inhibiting the serine proteases, thrombin, and
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plasmin. Plasmin has been shown to be a potent vasoconstrictor of canine basilar artery in vitro.31 Thrombin on the other hand, produces a significant contraction of canine basilar artery in vitro but significant tachyphylaxis develops, making it less likely that thrombin plays a role in prolonged vasospasm.31

It is interesting that heparin, which accelerates the formation of complexes of antithrombin III with various substrates, including thrombin and plasmin, has been recently proposed as a potential therapeutic agent for the treatment of vasospasm.3 In a recent experimental study, Kapp, et al.,14 demonstrated that heparin prevented the angiopathy seen following SAH, but the question of vasodilator activity was not addressed. It would be pertinent to determine whether heparin might enhance some of the effects of antithrombin III observed in our studies in vivo or in vitro. It is difficult to ascribe the in vitro vasodilator effects of antithrombin III observed in the present studies to inhibition of plasmin, thrombin, or similar proteins by antithrombin III. Alternatively, the known specificity of antithrombin III, an inhibition of serine proteases,4 makes a direct inhibitory effect on numerous disparate agonists such as KCl, 5-HT, uridine triphosphate, thrombin, or plasmin29-31 seem unlikely. It is possible that the vasodilatation seen with commercial antithrombin III is solely a result of other vasodilator substances contaminating the preparation such as fatty acids or thrombin. Opposing this view, however, is the observation that highly purified antithrombin III is more vasoactive than the commercial preparation (RP White, personal communication).

The endothelial independence of antithrombin III-induced vasodilatation was also demonstrated in vitro by White and Robertson.29-31 Other studies investigating antithrombin III affinity have demonstrated avid uptake to subendothelial tissue in addition to the well-documented endothelial binding.13 These findings all support a direct action of antithrombin III on the vascular media.

In light of the functional and perhaps structural endothelial damage seen in delayed arterial spasm,6,20 it is not surprising that many vasodilators which act by means of the release of endothelium-derived relaxing factor would be ineffective in treating this disorder. Furthermore, hemoglobin has been shown to be a selectively blocking endothelium-dependent vasodilatation.12 Therefore, even in the absence of endothelial cell injury, free hemoglobin arising from the perivascular clot may be preventing several physiological and pharmacological mechanisms of vasodilatation following SAH. In this setting, endothelium-independent substances such as antithrombin III, calcium channel blocking agents, and papaverine might have a greater potential to dilate vessels narrowed by early delayed cerebral vasospasm.

The concentration in CSF of a number of plasma proteins involved in coagulation and fibrinolysis has been studied.3,26 There is very little information, however, as to the time course of CSF plasma protein concentrations in relation to SAH, and no information regarding the antithrombin III concentration over time in CSF following SAH.

Antithrombin III is stable in biological fluids and is known to be present in a relatively high concentration in extravasated blood.29 It is possible, then, that endogenous antithrombin III, either by a direct vasodilator effect on vascular smooth muscle or via its inhibition of vasoconstrictor proteins such as plasmin or thrombin, may delay the onset of or diminish cerebral vasospasm. Study of antithrombin III concentration over time in CSF following aneurysmal hemorrhage would be of interest in this regard. The mechanisms by which antithrombin III exerts its nonspecific and probably direct vasodilator effect also warrant further investigation in vitro.

These empirical observations that antithrombin III can reverse vasospasm in the rabbit model suggests that further investigations in other models of SAH-induced arterial narrowing, notably the primate model which bears the closest similarity to the human condition, are warranted.

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