**In vitro** assay of contractile activities of spastic canine basilar artery and its surrounding blood clot

EIICHI TANI, M.D., AND SHOGO YAMAGATA, M.D.

Department of Neurosurgery, Hyogo College of Medicine, Hyogo, Japan

A prolonged vasospasm was produced in the canine basilar artery by injection of fresh autogenous arterial blood into the chiasmatic cistern. Homogenates of lyophilized normal and spastic canine basilar arteries, as well as of lyophilized blood clot around the spastic artery, were made, and their contractile activities were studied *in vitro*. The homogenates of the lyophilized spastic arteries usually induced a dose-dependent sustained contraction, whereas those of the lyophilized normal arteries often produced a dose-dependent transient contraction, and those of the lyophilized blood clot induced a dose-dependent transient or sustained contraction. The initial maximum contractions produced by the homogenates of the lyophilized normal and spastic arteries were significantly different in their values, suggesting the presence of a vasoactive agent in the spastic artery itself. Preliminary pharmacological analysis of the vasoactive agent was attempted using methysergide and phentolamine.

**KEY WORDS** - prolonged vasospasm - basilar artery - lyophilized spastic artery - lyophilized blood clot

The pathogenesis of prolonged vasospasm lasting for 2 or 3 weeks is still open to controversy. Since the cerebral artery demonstrating the prolonged vasospasm is usually surrounded by blood clot, a vasoactive agent inducing a prolonged vasospasm may be present in the spastic artery itself or in the clot around it. The contractile response of the cerebral artery *in vitro* has been used to identify and analyze the vasoactive agent. 2 7, 9, 10, 11, 20, 25, 28

In the present study, we attempt to examine the contractile activity of homogenates of lyophilized spastic artery and blood clot on the normal canine basilar artery *in vitro*. We are presenting preliminary information regarding the pathogenesis of prolonged vasospasm.

**Materials and Methods**

*Production of Prolonged Vasospasm*

Eight to 12 ml of fresh autogenous arterial blood were injected through a transorbital route into the chiasmatic cistern of adult dogs, 10 to 18 kg in weight, as reported previously. 29 Two days after this injection, vertebral angiography was carried out immediately before the sacrifice of each animal, and the diameter of the basilar artery was determined from the projection of angiograms on a screen. More than 25% reduction in caliber of the basilar artery was considered as evidence of prolonged vasospasm. 29

*Materials for In Vitro Study*

The animals with prolonged vasospasm were sedated with the intravenous administration of Nembutal (pentobarbital), and sacrificed by a rapid exsanguination. Immediately after removal of the brain, the blood clot around the spastic basilar artery was removed rapidly and completely under a Zeiss surgical microscope in a cold room. The blood clot and the spastic basilar artery were placed into liquid nitrogen and then lyophilized separately in a VirTis Unitrap II freeze dryer.

The normal basilar artery was similarly lyophilized. The lyophilized specimens were kept at $-20^\circ$ C until their contractile activities were tested *in vitro*. About 10 animals were needed to obtain 50 mg of the lyophilized materials of the spastic or the normal basilar arteries or the blood clot.

*In Vitro Study*

Normal adult dogs were anesthetized with the intravenous injection of Nembutal and sacrificed by rapid exsanguination. The brain with the basilar artery attached was placed in modified Krebs solution warmed to $37^\circ$ C and aerated with 95% $O_2$ and 5% $CO_2$. A segment of the basilar artery was obtained by sectioning the vessel with two razor blades fixed in parallel $3$ mm apart. The segment of the basilar artery
FIG. 1. Polygraph of a DPS-induced contraction of canine basilar artery in vitro. Although its amplitude is slowly decreased, the contraction is sustained and returns to the baseline resting tension with a washing with MKS. DPS: depolarizing solution; MKS: modified Krebs solution; RT: resting tension.

was then mounted on rigid parallel prongs in a chamber similar to those described by Nielsen and Owman and Allen, et al. The chamber was then filled with 5 ml of modified Krebs solution of the following composition: NaCl 118.9 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 14.9 mM, and dextrose 5.6 mM (pH 7.4), aerated with 95% O₂ and 5% CO₂, and warmed at 37 ± 0.5°C by means of a circulating temperature bath.

Isometric tension of the arterial segment in vitro was measured with a Nihon-Koden FD transducer with Nihon-Koden multipurpose polygraph. The arterial segment was allowed to stabilize at a resting tension of 200 to 400 mg for 1 hour and then increased to a resting tension of 3 gm before the start of the experiment. A trial contraction was made with a depolarizing solution (DPS) at the beginning of each experiment to determine the condition of the arterial segment, and values of contractions induced by the test materials were expressed as a percentage of maximum DPS response on the same arterial segment. Only those segments producing at least a 2-gm tension with DPS were used. The DPS was composed of 76 mM K₂SO₄, 10 mM KCl, 16 mM KHCO₃, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, and 5.6 mM dextrose. The test materials (the lyophilized normal and spastic basilar arteries, and the lyophilized blood clot) were homogenized in a mortar and then in a Wheaton homogenizer, and dissolved in modified Krebs solution before use. In order to obtain a dose-response relationship, the test materials were added in a cumulative manner to the bathing media in the chamber. To avoid foaming when the solution containing protein was added, a rotating magnetic stirring bar was used in the chamber. Serotonin, methysergide, and phentolamine were used for examination of pharmacological properties of vasoactive agents in the lyophilized test materials.

Results

Contractions Induced by Depolarizing Solution

Three arterial segments were used to determine the sensitivity of the artery, the rapidity of the response, and the accuracy of tension measurement with the polygraph tracing of the tension developed in response to DPS (Fig. 1). The arterial segment contracted rapidly, attaining a maximum tension within 2 minutes, which was usually sustained. The sustained contraction decreased slowly to about 82.4% of the maximum tension over 30 minutes, and, when washed with modified Krebs solution, returned to its baseline resting tension within 5 minutes of initiation of the washing procedure.

Dose-Dependent Responses to Test Materials

When the test materials were dissolved in modified Krebs solution, a small amount of sediment was formed in the bottom of the chamber during the experiment. Six arterial segments were tested with a cumulative addition of up to 10 mg/ml homogenates of lyophilized spastic arteries (HSA), and four arterial segments were used in dose-dependent responses to up to 10 mg/ml homogenates of lyophilized normal arteries (HNA) and of lyophilized blood clot (HBC) (Fig. 2). Arterial segments usually contracted in a cumulative dose-dependent manner to the test materials, but the values of contractions (expressed as a percentage of maximum DPS-induced contraction)
Contractility of spastic artery and blood clot varied from experiment to experiment. The dose-dependent responses to HNA and HSA were statistically different in their magnitude; \( p < 0.05 \) in doses of 6, 8, and 10 mg/ml, and \( p < 0.1 \) in doses of 2 and 4 mg/ml. The statistical difference of dose-dependent responses to HNA and HBC was \( p < 0.25 \) in doses of 4 to 10 mg/ml, and \( p < 0.5 \) in 2-mg/ml doses. No statistical difference was evident in dose-dependent responses to HSA and HBC. In addition, it was noted that a relaxation occurred in one experiment with 2, 4, 6, and 8 mg/ml HNA.

**Polygraph Tracing of Arterial Responses to Test Materials**

**Lyophilized Spastic Artery Homogenates.** Four arterial segments were used to analyze the arterial response to 10 mg/ml HSA. The response was usually a sustained contraction, as illustrated in Fig. 3. An initial maximum contraction, about 45.7% of maximum DPS-induced contraction, occurred within 3 minutes. Subsequently, during the first 8 minutes, the contraction decreased slowly to about 56.3% of the initial maximum tension. The arterial segment then slowly increased in tension and attained a sustained contraction, about 128.1% of its initial maximum contraction, for at least 2 hours. After the sustained contraction, the arterial segment did not return to its baseline resting tension when washed with a large amount of modified Krebs solution, but did so promptly when washed in a Ca-free modified Krebs solution, which contained 4 mM ethyleneglycol-bis-(\( \beta \)-amino-ethyl ether) N,N-tetraacetic acid (EGTA).

**Lyophilized Normal Artery Homogenate.** The polygraph tracings of four arterial segments in response to 10 mg/ml HNA were more complicated than those in response to 10 mg/ml HSA. The upper tracing in Fig. 4 showed an initial maximum contraction, about 22.4% of maximum DPS-contraction, within 4 minutes and then gradually returned to or even below its baseline resting tension, thus demonstrating a transient contraction. This type of tracing was found in three experiments. In another experiment (Fig. 4, *lower tracing*), the arterial segment increased slowly in tension about 9 minutes after the return to baseline resting tension and attained about 40% of maximum DPS-induced contraction, four times its initial maximum contraction, about 38 minutes after the addition of HNA, and did not return to its baseline resting tension with the washing procedure.

**Lyophilized Blood Clot Homogenate.** The responses of five arterial segments to 10 mg/ml HBC were not as consistent as those to 10 mg/ml HSA. The upper tracing in Fig. 5 exhibited a prompt contraction, about 26.6% of maximum DPS-induced contraction, and lasted only for about 5 minutes. There was no evidence of further contraction. This type of response was seen in three experiments, and was often found in experiments with less than 10 mg/ml HBC. The lower tracing in Fig. 5 shows an initial maximum contraction, about 45.2% of maximum DPS-induced contraction, within 1 minute, which diminished slowly to about 42.1% of the initial maximum contraction in 13 minutes, and reached a sustained contraction. The sustained contraction did not return to the baseline

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resting tension with washing. A sustained contraction was observed in two experiments.

The characteristics of the polygraph tracings of arterial responses to the test materials are summarized in Table 1.

**Effects of Methysergide and Phentolamine on HSA-Induced Contraction**

Methysergide has been found to block 5-HT receptors, particularly on the smooth muscle. Allen, *et al.*, found that 10⁻⁴ M methysergide reversibly blocked the basilar artery's response to maximum serotonin contraction. The experiment was conducted with two arterial segments to determine if contractions induced by serotonin or HSA decreased to baseline resting tension in response to the addition of methysergide. As illustrated in the upper tracing of Fig. 6, a maximum contraction induced by 5 × 10⁻⁷ M/ml serotonin returned within 1 minute to the baseline resting tension in response to the addition of 10⁻⁴ M/ml methysergide. On the other hand, the contraction produced by 10 mg/ml HSA, when 10⁻⁴ M/ml methysergide was added, was reduced by about 21.1% within 1 minute and by about 31.6% in 4 minutes, and did not return to the baseline resting tension thereafter (Fig. 6, lower tracing). The sustained contraction induced by HBC also was reduced by

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**TABLE 1**

*Characteristics of polygraph tracings of arterial responses to test materials*

<table>
<thead>
<tr>
<th>Test Materials</th>
<th>Magnitude of Initial Contraction</th>
<th>Type of Contraction</th>
<th>Washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPS</td>
<td>100%</td>
<td>sustained</td>
<td>immediate return to resting tension</td>
</tr>
<tr>
<td>HNA (10 mg/ml)</td>
<td>18.4 ± 9.4%†</td>
<td>usually transient</td>
<td></td>
</tr>
<tr>
<td>HSA (10 mg/ml)</td>
<td>72.8 ± 31.6%‡</td>
<td>sustained</td>
<td>no return to resting tension</td>
</tr>
<tr>
<td>HBC (10 mg/ml)</td>
<td>50.5 ± 30.4%‡</td>
<td>transient or sustained</td>
<td>no return to resting tension in sustained contraction</td>
</tr>
</tbody>
</table>

*†Mean = standard deviation. Statistical differences of HNA- and HSA-induced contractions and HNA- and HBC-induced contractions are p < 0.05 and p < 0.25, respectively.*

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**FIG. 4.** Polygraphs showing two types of canine basilar artery contractions induced by homogenates of lyophilized normal arteries (HNA). The contraction returns spontaneously to and then below the baseline resting tension (upper). *Lower Tracing:* The basilar artery shows a transient contraction and then slowly develops a sustained contraction. The sustained contraction is not decreased with a washing with modified Krebs solution (MKS). DPS: depolarizing solution.

**FIG. 5.** Polygraphs of two types of canine basilar artery contractions induced *in vitro* by homogenates of lyophilized blood clot (HBC). *Upper Tracing:* Initial maximum contraction, followed by a sustained contraction. The sustained contraction is not decreased with a washing with modified Krebs solution (MKS). DPS: depolarizing solution.
Fig. 6. Polygraphs of a sustained contraction induced by $5 \times 10^{-7}$ M serotonin (5-HT) (upper tracing) rapidly returning to the baseline resting tension in response to $10^{-4}$ M methysergide (MS). Contraction of canine basilar artery induced by homogenates of lyophilized spastic arteries (lower tracing) is slightly reduced when $10^{-4}$ M methysergide is added.

Fig. 7. A contraction of canine basilar artery induced in vitro by homogenates of lyophilized spastic arteries is moderately reduced in response to $1.3 \times 10^{-4}$ M phentolamine. DPS: depolarizing solution; MKS: modified Krebs solution.
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about 30.8% with the addition of 10^-4 M/ml methysergide.

Phentolamine is an α-adrenergic blocking agent and also inhibits response to serotonin.5,34 Two experiments were carried out to examine the effect of phentolamine on HSA-induced contraction. Figure 7 is a typical tracing of the effect of phentolamine on an HSA-induced contraction. When 1.3 × 10^-4M/ml phentolamine was added, the contraction was reduced by about 48.2% within 4 minutes and then slowly decreased by about 79.3% in 30 minutes.

Discussion

The amount of the initial maximum contractions induced by HNA and HSA was significantly different, particularly in doses of 6 to 10 mg/ml. The response to HNA was often a transient contraction, whereas HSA usually induced a sustained contraction which was not decreased with washing. This characteristic feature seems to be similar to the clinical behavior of prolonged vasospasm, suggesting that HSA is tightly bound at its arterial receptor to induce a sustained contraction. The structural differences shown by spastic arteries include a rapid loss of intraneuronal catecholamine fluorescence, a prolonged inhibition of the neuronal uptake and retention of norepinephrine, and evidence of myonecrosis, intercellular granules, and vesicles. It may be suggested, therefore, that a vasoactive agent is contained in the spastic artery itself, possibly in the necrotic muscle cells, or the intercellular granules and vesicles. The great variability in the amplitude of HSA-induced contractions may be partly due to the amount of the vasoactive agent in the spastic artery used in the experiment, and partly due to the sensitivity of the arterial segment to the vasoactive agent. The delayed, sustained contraction after a transient response in one experiment with HNA (Fig. 4, lower tracing) could be ascribed to the occasional presence of a small amount of myonecrosis and intercellular granules and vesicles as a result of normal metabolism in the basilar artery.

The amount of initial maximum contractions induced by HSA and HBC showed no statistical difference, although those induced by HBC were usually greater than those induced by HBC (Table 1). The HBC-induced contraction was transient or sustained if 10 mg/ml were used and often transient with less than 10 mg/ml. Contractile activity has been reported in hematomat material from a ruptured aneurysm, in hemolyzed materials, and in cerebrospinal fluid (CSF) from patients following subarachnoid hemorrhage. Serotonin or breakdown products of erythrocytes have been reported as vasoactive agents. Buckell indicated no correlation between the presence of vasospasm and the content of serotonin in CSF around the ruptured aneurysm. The platelets in the blood clot associated with prolonged vasospasm were usually devoid of 5-HT granules as suggested by Mills et al. and by our own observations, and the addition of 10^-4 M methysergide did not cause the sustained HBC-induced contraction to return to the baseline resting tension in these experiments. On the other hand, serotonin- or DPS-induced contraction returned readily to the baseline resting tension when washed. In addition, electron microscopy of HBC demonstrated a homogeneous electron-dense material, which was quite similar in appearance to intact erythrocytes, suggesting the presence of breakdown products of erythrocytes. It may be suggested, therefore, that HBC-induced contraction is not due to serotonin liberated from the platelet but to breakdown products of erythrocytes.

The breakdown products of erythrocytes are presented in smaller amounts in situ than in HBC, because HBC was produced by the mechanical homogenization of the blood clot. Consequently, the spontaneous breakdown products of erythrocytes, particularly if washed away by CSF, may not produce a sustained contraction. However, it is necessary to determine if the breakdown product in HBC is the same as occurs in situ 2 days after subarachnoid hemorrhage. If a sustained contraction occurred with HBC, it was not decreased with washing as was observed in the sustained HSA-induced contraction. In this respect, the sustained HBC-induced contraction is similar to prolonged vasospasm seen in the clinical setting.

The occasional prolonged vasospasm after aneurysm surgery without the addition of subarachnoid blood may not be caused by the breakdown products of erythrocytes. Experimental intracisternal blood injection does not always produce a prolonged vasospasm, although an early vasospasm does occur and a large amount of blood clot surrounds the basilar artery. Similarly, prolonged vasospasm in patients does not necessarily follow every subarachnoid bleeding. An experimental prolonged vasospasm occurs more easily and severely with the rupture of an artery than with a subarachnoid injection of blood. Sustained contraction was shown in these experiments to be more severe in HSA than in HBC. All of these facts may suggest that the arterial factor plays a more important role in the production of prolonged vasospasm than does the blood clot, and is present as a vasoactive agent in the spastic artery itself as shown in the experiment with HSA.

Since the smooth-muscle cells in the canine basilar artery are joined together with gap junctions, a vasoactive signal may be transmitted rapidly through the gap junction from one muscle cell to another, and a chain of smooth-muscle cells contract simultaneously and continuously to produce the prolonged vasospasm. Thus, the vasospasm may occur in arteries not only near the ruptured aneurysm but also at some distance from it and free of the blood clot.

The preliminary pharmacological examination showed that the sustained HSA-induced contraction
was decreased slightly by $10^{-4}$ M methysergide, and moderately by $1.3 \times 10^{-4}$ M phentolamine, both of which block completely the basilar artery’s response to serotonin.\textsuperscript{3,7} The difference in the response of the sustained contraction to methysergide and phentolamine may be due to the pharmacological property as an $\alpha$-adrenergic blocking agent and other functions of phentolamine.\textsuperscript{4,6} An $\alpha$-blocking agent has been shown to block or reverse early vasospasm following the subarachnoid injection of blood.\textsuperscript{19-16,20,27,28} However, it was noted that sustained contraction induced by HSA usually did not return to the baseline resting tension in response to $1.3 \times 10^{-4}$ M phentolamine, which is characteristic of prolonged vasospasm. The chemical or pharmacological nature of the vasoactive agent in HSA is unknown at present, and can only be inferred from the response of its sustained contraction to methysergide and phentolamine. This problem needs further detailed analysis.

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Address reprint requests to: Eiichi Tani, M.D., Department of Neurosurgery, Hyogo College of Medicine, Mukogawa-cho, Nishinomiya, Hyogo, Japan 663.