An In Vitro Study of Prolonged Vasospasm of a Rabbit Cerebral Artery

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SUMMARY Longitudinal stretch of the rabbit basilar artery produces local injury followed by prolonged circular constriction. After stretching and rapid release in vitro localized constrictions promptly occurred. This could be prevented by prior treatment with cyanide or calcium-free solution. Once produced, constrictions persisted for more than 72 hours. Previously induced constriction was not reversed by treatment for two hours with cyanide or by removing calcium. Histological observation indicated that constricted areas were associated with a discrete circumferential rupture of the internal elastic lamina and disruption and thinning of the underlying media.

Most workers agree that release of vasoactive substances from subarachnoid blood is a major cause of diffuse cerebral vasospasm after rupture of an intracranial aneurysm. Yet there is experimental evidence that spasm may be more prolonged and severe when puncture of a cerebral vessel is combined with injection of blood into the subarachnoid space than when blood is applied without injury. Studies of mechanical stimulation of cerebral vessels have found the resulting constriction to be short lived, lasting less than 30 minutes. It is this failure to produce local injury followed by prolonged discrete circular constriction. The accompanying histological changes are described, and the role of smooth muscle in the production of constriction is confirmed. These findings demonstrate that under certain conditions, such as injury, cerebral arterial smooth muscle may undergo an essentially irreversible contraction. Such a mechanism could contribute to the etiology of prolonged cerebral vasospasm after SAH or traumatic injury to the cerebrum.

Specific catecholamine fluorescence at the adventitio-medial junction was unchanged in constricted areas. The relationship between smooth muscle cell length and resting tension of artery segments with and without constrictions was compared. Segments with constrictions had a shorter muscle length for any given resting tension, which confirms that constriction was not due to passive collapse of the vessel wall. These findings suggest that injury of cerebrovascular smooth muscle may result in essentially irreversible vasospasm. Such a mechanism could contribute to the pathogenesis of prolonged cerebral vasospasm after SAH or traumatic injury.

A preliminary report of a portion of this work has been presented at the Second International Symposium on Vascular Neuroeffector Mechanisms, Odense, Denmark.

Methods

New Zealand white rabbits (weight 2 to 3 kg) were stunned by a blow on the nose and bled from the neck. The entire brain with attached arachnoid membrane and blood vessels was removed and placed in Krebs bicarbonate solution at room temperature containing (mM): Na+ 144.2, K+ 4.9, Ca2+, 1.3; Mg2+, 1.2; Cl−, 126.7; HCO3−, 25.0; SO4−, 1.19; ethylene diamine tetraacetic acid, 0.027; and glucose, 11, and bubbled with 95% O2 and 5% CO2. The basilar artery was observed and further dissection was carried out using a Wild stereomicroscope. Photographs were taken with a 35-mm camera attachment. In some experiments NaCN (200 mg per liter) was added to the Krebs solution and glucose was omitted. In other experiments calcium was omitted from the Krebs solution and 2 mM EGTA (ethyleneglycol-bis [β-amino ethyl ether] N,N-tetraacetic acid) was added.

HISTOLOGY

After dissection and diagrammatic recording of the presence and site of areas of constriction, the basilar artery was cut open longitudinally and laid flat on a small card. This was then fixed in buffered formaldehyde, dehydrated and embedded in paraffin. Sections were prepared for light microscopy and photographed with a Wild stereomicroscope. The adventitia and internal elastic lamina were stained for elastic tissue with toluidine blue, periodic acid Schiff (PAS), and van Gieson's elastic stain. Sections from all areas of constriction were examined and compared with areas of nonconstriction.
and embedded in paraffin. Longitudinal 6-μm serial sections were made and stained with Verhoeff's elastin stain.

**FLUORESCENCE MICROSCOPY**

Whole mounts of the basilar artery were processed for fluorescence microscopy by the glyoxylic acid method. Some arteries were prepared for fluorescence microscopy by freeze-drying using the Falck-Hillarp technique for catecholamines. Sections of 5 μm, either transverse or longitudinal, were examined under a Zeiss fluorescence microscope.

**LENGTH-RESTING TENSION CURVES**

Ring segments (length, 2 mm) of the basilar artery were cannulated with two platinum wires and mounted in an isolated tissue bath in Krebs solution at 37°C bubbled with 95% O₂ and 5% CO₂. One of the wires was attached to a movable arm for adjustment of rest tension, while the other wire was connected to the arm of a Statham force transducer (G 10B±0.15 Oz) in order to monitor isometric force via a Grass polygraph. Ring segments with constrictions were paired with segments without constrictions from the same artery. After a one-hour equilibration period, each ring segment was stretched to a resting tension of 0.05 g, and a further equilibration period of one hour followed. Direct observation with a dissecting microscope and calibrated eye-piece was then periodically made to measure the distance between the two cannulating wires. Since the arterial ring is stretched between these two wires, the two walls of the ring are parallel and close together. In this vessel the smooth muscle is arranged in a circular fashion so that the distance between the two wires is directly related to the length of the circular smooth muscle cell layer. After equilibration at 0.05 g rest tension, the distance between the two cannulating wires was observed. Each ring was then slowly stretched to a series of given resting tensions, allowing at least 15 minutes' equilibration at each new level. The distance between the cannulating wires was observed at each level of resting tension as a measure of the length of the smooth muscle cell layer.

**STATISTICS**

Student's t-test for paired values was used. Standard errors are reported throughout.

**Results**

**PRODUCTION OF PROLONGED CONSTRICTION**

Discrete, localized constrictions of the basilar artery could be produced in vitro when a longitudinal stretch was applied. When the basilar artery was stretched to twice its initial length and then released, localized constrictions rapidly occurred. These could be seen developing to completeness in less than 10 seconds. Constrictions occurred at any point along the length of the vessel, giving the basilar artery a beaded appearance (fig. 1). The exact location and number of constrictions found after stretch varied from vessel to vessel.

After constrictions were formed they persisted as long as observed, for up to 72 hours when kept in Krebs solution at room temperature or at 4°C. Previously formed constrictions were not reversed after incubation with Krebs solution containing cyanide for up to two hours, nor were they reversed by removing calcium from the Krebs solution and adding 2 mM EGTA.

**HISTOLOGY**

When the histological sections were compared with the initial observations of the sites of the apparent constrictions, it was found that they were associated with a discrete circumferential rupture of the internal elastic lamina of varying extent, sometimes involving the entire circumference (fig. 2). The frayed ends of the elastic lamina projected into the lumen of the artery. Localized disruption and thinning of the underlying media, variable in depth, were seen only where the internal elastic layer was absent. Smooth muscle cells were seen to be distorted at this site and frequently elongated in a longitudinal plane.

The arterial wall was also distorted and angulated inward, so that collagen bundles of the adventitia were separated at the site of rupture of the internal elastic lamina. No consistent change was seen in smooth muscle cells adjacent to the lesion.

**FLUORESCENCE HISTOLOGY**

Two methods were utilized to observe specific catecholamine fluorescence of constricted areas. Freeze-
dried vessels were reacted with paraformaldehyde and sectioned longitudinally (six basilar arteries; 15 constrictions), and whole mount preparations using the glyoxylic acid technique (one basilar artery; 12 constrictions) were studied. Using either method, the specific catecholamine fluorescence was still present in the constricted region of the vessel, as compared with surrounding areas (fig. 3). Due to autofluorescence of elastic elements, the breaks in the internal elastic lamina could also be seen. Bright yellow punctate fluorescence in the vessel lumen was found to be adherent to areas where the elastin was deficient. This can be attributed to the serotonin content of platelets which would be expected to attach themselves to the exposed collagen in the injured vessel wall.14

MUSCLE LENGTH-REST TENSION RELATION

The relationship between smooth muscle cell length and tension of basilar artery ring segments with and without constrictions was compared (fig. 4). Weights of ring segments with and without constrictions were not significantly different (P > 0.4). At low tensions, the muscle length of ring segments with one or more localized constrictions was significantly smaller (P < 0.001) than the length of segments without constrictions. At larger rest tensions the two became similar. When tension was subsequently decreased, segments with constrictions no longer had a significantly smaller length. Thus, stretching the vessel reversed the constriction. Direct observation of these ring segments after such stretch showed that localized constrictions were no longer present although the ring of broken internal elastic lamina could still be seen.

PREVENTION BY METABOLIC INHIBITION

Segments of the basilar artery were incubated in Krebs solution at 20°C or 37°C for one to four and one-half hours. After incubation, 23 segments were stretched and 31 discrete breaks in the internal elastic lamina were produced followed by localized constriction. Constriction was complete in less than 10 seconds. When artery segments were treated with Krebs solution containing cyanide, no glucose and no oxygenation for one-half hour, the formation of constrictions after stretch was not prevented but was markedly slowed. In this case constriction was not complete until 20 seconds after the application of stretch. Nineteen artery segments were pretreated for one and one-half to three hours with Krebs solution containing cyanide, no glucose and without oxygenation. After stretch, 41 discrete breaks in the internal elastic lamina could be seen which extended more than halfway or completely around the circumference of the vessel wall. However, after such prolonged pretreatment with cyanide, only slight localized constriction was occasionally seen; usually there was no constriction at the site of the injury.

PREVENTION BY REMOVAL OF CALCIUM

Three basilar arteries were incubated for two hours in Krebs solution with no calcium added and with EGTA. After longitudinal stretch, 15 breaks in the internal elastic lamina were produced, but there was no constriction at the site of the injury.

Discussion

Vasospasm has been described as “contraction of a muscular vascular tube, which causes great narrowing of the lumen, which occurs in response to stimuli from an abnormal physiological state, and which differs in some significant degree from physiological contraction in the vessel.”15 Thus, all vasoconstriction is not spasm; only when the prolonged and severe nature of the constriction is explained can any theory of spasm be justified.

Release of vasoconstrictors from subarachnoid blood has been postulated to produce cerebral vasospasm.14-18 It has also been suggested that spasm is mediated by traumatic release of norepinephrine from vascular nerves.15,20-22 The particular agent or agents responsible remains in question, partly because the specificity of blocking agents used, particularly phenoxybenzamine, has not always been demonstrated. Undoubtedly release of vasoactive substances from the blood can play a role in the genesis of cerebral
vasospasm; release of substances from cerebral vascular nerves or from ischemic brain parenchyma may also contribute.

In addition to the role of vasoconstrictor substances in the genesis of cerebral vascular spasm there is evidence to suggest that injury also may be important. Consistently it has been noted that spasm induced by puncturing a cerebral artery is more prolonged and severe than spasm produced by injecting blood into the subarachnoid space. For example, in rhesus monkeys spasm produced by the injection of 3 ml of fresh blood lasted one to three days, while spasm produced by a needle puncture of the intradural carotid artery lasted more then seven days. This suggests that mechanical trauma to the vessel itself may contribute to the etiology of spasm, although there could be other explanations of this difference. There is also evidence that widespread necrosis of cerebral arterial smooth muscle cells is associated with the vasospasm that occurs after cerebral arterial puncture or the injection of vasoactive substances into the cerebrospinal fluid. It has been suggested that injury to smooth muscle cells produced by vasoactive substances produces prolonged constriction either by direct structural changes or by the release of biologically active substances from injured cells, causing neighboring undamaged cells to contract.

It has been shown repeatedly that mechanical stimulation of cerebral vessels produces a contractile response which invariably lasts less than 30 minutes. Indeed, we have demonstrated a similar short-lived response to mechanical stimulation in the rabbit basilar artery in vitro (unpublished observation). That injury after longitudinal stretch can produce long-lived constriction of a cerebral artery is a new finding. In fact, this has not been reported to our knowledge in any type of blood vessel. The constriction produced by stretch-induced injury demonstrated in this paper persists unchanged for up to 72 hours, does not require oxygen, and is not reversed by poisoning with cyanide or by removal of calcium. This constriction is accompanied by characteristic histological changes: the internal elastic lamina is ruptured in a ring around the vessel, and there is damage to the media as well as to the adventitia. The rupture of the elastic lamina appears to precede constriction; after short treatment with cyanide these processes are separated enough in time to see that the constriction occurs separately. The discrete breaks in the elastic lamina must occur at some weak point in the vessel wall, since in some vessels after stretch, no such breaks are formed, while in other vessels, after some breaks are formed, further stretch does not produce more. It is of interest that rupture of the internal elastic lamina and loss of cellular detail in the media also have been reported after in vivo mechanical stimulation of cerebral arteries in the baboon. Although these histological changes were not related to prolonged constriction of the vessel, prolonged changes in responsiveness to arterial PCO2 were observed.

The prolonged constriction described in the present study does not seem to be caused by release of vasoactive substances either from intramural nerves or from adherent platelets. Fluorescent studies indicated that catecholamines in the nervous plexus were not altogether depleted in constricted areas, although some loss could have occurred. Although platelets were seen to adhere to areas where the intima was disrupted, this does not seem to account for constriction. Even when blood was not present in the lumen, when arterial segments were stretched after prolonged incubation in Krebs solution, severe localized constrictions were still produced.

This prolonged localized constriction could be due to three possible causes: There may be a passive collapse of the wall due to rupture of the internal elastic lamina, a plastic deformation of the vessel wall, or an active contraction of smooth muscle on either side of the disrupted area. Two types of experiments were designed to distinguish between these causes. First, the resting length-tension relationship of paired ring segments was studied. Those segments with constrictions had a smaller smooth muscle cell length for any given tension than segments without constrictions. Such a finding rules out a passive collapse of the vessel wall, since passive collapse would lead to a greater distensibility. As tension was increased, the difference in length of the smooth muscle cell layer between segments with and without constrictions was abolished. This can be attributed to a disruption of contractile links by excess stretch. In fact, these segments were no longer visibly constricted after such stretch. The second experiment involved depletion of ATP by treatment with cyanide. Cyanide did not prevent the break in the elastic lamina, but constriction was prevented. This supports the hypothesis that constriction results from an irreversible contraction of smooth muscle, although a plastic deformation of the vessel wall that is also influenced by cyanide cannot be ruled out. However, there are no published data to support the likelihood of such a possibility. The observation that constriction can occur several seconds after the stimulus (longitudinal stretch) has been completed and the break in the elastic lamina is formed also favors the hypothesis of an irreversible contraction of smooth muscle. Furthermore, we have found that removal of calcium from the bathing medium, which is known to prevent smooth
muscle contraction, prevents the formation of constrictions after longitudinal stretch.

The mechanism of such an irreversible contraction of vascular smooth muscle after stretch-induced injury is unknown. It is possible that injured cells release a biologically active substance that causes adjacent undamaged cells to contract. While this might explain the origin of the contraction, it does not account for its persistence nor its resistance to treatment with cyanide or removal of calcium.

There is little information in the literature to suggest a probable mechanism to explain these properties. Rigor mortis of skeletal muscle is one possible model, but the characteristics of rigor mortis are different from those observed in the present study. Rigor mortis is a stiffening and loss of extensibility of skeletal muscle which involves relatively little shortening of contractile elements. Furthermore, rigor mortis occurs with depletion of ATP and is prevented by supplying oxygen to the tissue. The irreversible contraction we have studied is prevented by depletion of substrate and involves an appreciable change in length.

Another possible model for a prolonged contraction is the catch mechanism of certain molluscan muscles and the hysteretic properties of contraction in mammalian skeletal muscle. Although the time course of these catch mechanisms is long in relation to the normal time course of stimulated contraction, these mechanisms demonstrate decay in a matter of minutes. The catch property is thought to be due either to some property of the contractile proteins or to the calcium binding mechanisms. The prolonged constriction produced in the basilar artery after stretch-induced injury may be due to some type of catch mechanism; perhaps the process of relaxation has in some way been prevented. Once this catch mechanism is in operation, it seems that it can be overcome only by mechanically breaking the catch linkages by increasing the vessel diameter.

Although longitudinal stretch of the magnitude described above may not occur in vivo the importance of this work lies in the demonstration that injury to the vessel wall may lead to a prolonged and essentially irreversible contraction. This suggests that, when associated with injury, contraction may not be initiated by reversible physiological or pharmacological mechanisms but by an abnormal route resulting in an irreversible contracture. Cell damage produced by mechanical trauma, by vasoactive substances, or by other causes, if it leads to such an irreversible contracture, could certainly be important in the genesis of cerebral vasospasm after subarachnoid hemorrhage or traumatic injury.

References

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