Does Cerebral Vasospasm Result From Denervation Supersensitivity?

Thomas A. Duff, John A. Feilbach, and Grayson Scott

This study examined the role of denervation supersensitivity in the development of cerebral vasospasm. Adrenergic denervation of cat basilar artery was accomplished by resection of the superior cervical ganglia or by injection of 6-hydroxydopamine into the cisterna magna. In vivo dose–response characteristics were determined for normal and for denervated arteries, and no significant differences were found between topical applications of serotonin, norepinephrine, epinephrine, fresh blood, or incubated blood. In addition, analysis of cat blood incubated in vitro revealed that the levels of serotonin, norepinephrine, and epinephrine diminished over time, whereas levels of hemoglobin and methemoglobin increased up to Day 14. The results of this study indicate that adrenergic denervation is not the cause of cerebral vasospasm and that, whatever the mechanism, hemoglobin is far more likely to play a role than are the other agents. (Stroke 1987;18:85-91)

The proposal that denervation supersensitivity plays a role in the development of cerebral vasospasm (CVS) has received intermittent but nonetheless tantalizing corroboration. Symon was the first to suggest that the extended time course of CVS might reflect underlying denervation,1 and the subsequent discovery that catecholamine histofluorescence was diminished in cerebral arteries subjected to experimental subarachnoid hemorrhage (SAH) added a measure of morphological support.2,3 Recently, however, direct anatomical evidence of SAH-induced damage to neural elements of cerebral vessels has been obtained by electron microscopy.4 Moreover, several investigators have detected apparent pharmacological substrates of supersensitivity in cerebral vessels following SAH.5-7 Tsukahara et al., for example, reported that SAH resulted in an increase of both the equilibrium dissociation constant and the maximal binding of norepinephrine to the \( \alpha \) receptors in human cerebral arteries.8 In spite of these findings, there has been no definitive evidence that SAH produces supersensitive responses in cerebral vessels. Perhaps much of the ambiguity is understandable in view of the conflicting findings on the effects of denervation itself.9-13

Because the role of denervation supersensitivity in CVS has remained unclear and because much of the evidence has been based on in vitro measurements, the present study was undertaken to ascertain whether adrenergic denervation alters the in vivo responses of the cat basilar artery. Examination of dose–response characteristics was made not only for natural neurotransmitter agents, but also for fresh and for incubated blood, both of which have vasoconstrictor activity.

Materials and Methods

Adrenergic Denervation of Basilar Artery

Twenty-two mongrel cats weighing between 4 and 6 kg were used in this study. In 5 animals the basilar arteries were surgically denervated of their adrenergic input by bilateral resection of the superior cervical ganglia, performed with the animal under halothane + N\(_2\)O anesthesia (see below). Successful sympathectomy was evident from the immediate and sustained appearance of the nictitating membranes. In 3 other animals chemical denervation of the basilar artery was accomplished by injection of 5 mg 6-hydroxydopamine into the cisterna magna.7,14-16

Transclival Exposure of Basilar Artery

Anesthesia was induced with 3:1 N\(_2\)O:O\(_2\) and halothane at 5%. An endotracheal tube was inserted, and anesthesia was maintained with a Harvard ventilator using 1.5% halothane. Indwelling catheters were inserted into a femoral vein and artery, and throughout the experiment arterial P\(_{\text{CO}}\(_2\), P\(_{\text{O}}\(_2\), and pH were measured every 15-20 minutes on an IL-513 blood gas analyzer and maintained at between 37 and 43 torr, 150 and 200 torr, and 7.35 and 7.45, respectively. Blood pressure was monitored with a pressure transducer and continuously recorded. Five percent dextrose in water was administered intravenously to maintain systolic blood pressure between 100 and 120 mm Hg. Animal body temperature was maintained at 37° C with a heating pad. After achieving surgical anesthesia, a midline incision was made in the upper third of the neck and a paratracheal approach was made to the clivus. With the aid of an operating microscope, the clivus was removed, the dura incised, and the arachnoidal membrane opened by sharp dissection. Between application of test solutions, the artery was covered with Ringer's solution at 37° C.

Determination of Dose–Response Characteristics

The dose–response characteristics of basilar arteries were determined in 4 control animals and in 8 animals...
whose arteries had been surgically or pharmacologically denervated 7 days earlier. Test substances were made up in Ringer's solution, which included Ca++ at 2.5 mEq/l. Each solution was applied for 30 seconds, removed, reapplied for 90 seconds, removed, and then reapplied for 60 seconds. At that time, the solution was suctioned away, and a photograph immediately taken.

The ranges in concentration of test substances were serotonin (5-HT) from $10^{-4}$ to $10^{-7}$ M, norepinephrine and epinephrine from $10^{-2}$ to $10^{-7}$ M, fresh blood and 7-day incubated blood from full strength to a dilution of 1:1000. Basilar artery diameter was measured from Polaroid photographs taken through the operating microscope at 7.67×. For each artery the diameter was the mean width of the blood column at 3 predetermined locations. Constriction or dilation was calculated as percent of the arterial diameter immediately before application of the particular solution. Sufficient time for the artery to return to baseline caliber was allowed between applications.

Electron Microscopy

Following physiological experiments, electron microscopy (EM) analysis was carried out on 2 control, 2 surgically denervated, and 2 chemically denervated basilar arteries. Prior to removal of the artery, animals were perfused through the heart with 500 ml of 0.10 M phosphate buffer, pH 7.4, followed by 1,000 ml of 2% gluteraldehyde and 1% paraformaldehyde in 0.10 M buffer. The basilar artery was excised, washed in buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Durcupan. Ultrathin sections were counterstained with 1% lead citrate and 1% uranyl acetate. Sections were then examined on either a JEOL 100-CX or Hitachi H-600 electron microscope.

Measurement of 5-HT, Norepinephrine, Epinephrine, Hemoglobin, and Methemoglobin in Incubated Blood

The concentration of 5-HT, norepinephrine, and epinephrine in cat blood stored at 37° C was measured using high pressure liquid chromatography (HPLC). Blood samples from 5 cats were drawn aseptically through a 19-gauge needle inserted into a jugular vein. The blood was immediately transferred to 5-ml sterile collection tubes and allowed to clot and retract. After various incubation times, samples were spun at 1200g for 10 minutes. For measurement of catecholamines, the supernatant was removed and catecholamines adsorbed at pH 8.0 onto acid alumina (Fisher Scientific). The alumina was washed with water, and the catecholamines eluted off with 0.1 N HClO₄. Chromatography was performed with a Spectra Physics HPLC, using a reversed-phase Altex Ultrasphere Ion Pair C₈ column (25 × 0.46 cm i.d.). The mobile phase was 1:15 v/v methanol and 0.1 M potassium phosphate buffer at pH 3.0, 0.2 mM sodium octyl sulfonate (Kodak), and 0.1 mM EDTA. The column eluent was monitored with an electrochemical detector (Model LC-4A, Biochemical Systems, Inc.) and a glassy carbon electrode at a potential of +0.72 V vs. an Ag–AgCl reference electrode. Other chromatographic conditions were: flow rate, 1.0 ml/min; injection volume, 50 μl; internal standard, 3,4-dihydroxybenzylamine (DBA).

For measurement of 5-HT, blood was collected from 5 cats, stored, and incubated as described for the catecholamine assay. One milliliter of serum was deproteinized with 0.10 ml 4 M HClO₄, and an internal standard (0.02 ml 1.12 mM DBA) was added. After centrifugation at 27,000g for 15 minutes, the supernatant was decanted, and 0.25 ml 3 M NaOH and 1 ml 0.05 M ammonium acetate buffer were added. After filtration through a Millipore Swinnex assembly (filter pore size 0.45 μm), the extract was injected into the HPLC. Other parameters were as described above except that the mobile phase was 1:19 v/v methanol and 0.5 M ammonium acetate, pH 5.1.

Spectrophotometric determination of total hemoglobin was made following procedures described in Sigma Technical Bulletin No. 5257; methemoglobin concentration was measured by the method of Leahy and Smith.

Results

Dose–Response Characteristics in Control and in Denervated Arteries

The dose–response curves of control basilar arteries to application of 5-HT, norepinephrine, epinephrine, fresh blood, and incubated blood are shown in Figures 1 and 2. The ED₅₀ for 5-HT, norepinephrine, and epinephrine were approximately $2 \times 10^{-6}$ M, $7 \times 10^{-5}$ M, and $2 \times 10^{-4}$ M, respectively. In absolute terms, the greatest constriction occurred after application of undiluted supernatant from incubated blood.

As evident from Figures 1 and 2, there was a relatively small difference between the dose–response properties of control and of denervated cat basilar arteries to any of the test substances. It appeared, in fact, that denervation slightly diminished the degree of constriction to catecholamines as well as to fresh and to incubated blood.

EM of Denervated Arteries

Vessels removed from animals that had undergone either resection of the superior cervical ganglia or cisternal injection of 6-hydroxydopamine showed classical ultrastructural features of degeneration in sympathetic fibers and varicosities. Electron-dense accumulations were present within the axoplasm of numerous unmyelinated fibers, and nearly all varicosities containing dense-core vesicles were disrupted (Figure 3).

Levels of 5-HT, Norepinephrine, Epinephrine, Hemoglobin, and Methemoglobin in Fresh and in Incubated Blood

As indicated in Figure 4 (left), the concentrations of 5-HT, norepinephrine, and epinephrine in blood incubated in vitro at 37° C fell rapidly during the first 24 hours. The concentrations of hemoglobin and methemoglobin, on the other hand, rose steadily for 7 days, then changed minimally until day 14 (Figure 4, right).
Discussion

In Vitro vs. In Vivo Measurements of Dose-Response Characteristics

The dose-response characteristics of cerebral arteries have most commonly been determined using contraction chamber measurements of excised vessels. Although such determinations have provided invaluable information about the comparative effects of agonists and antagonists, it is risky to assume that the absolute values obtained from in vitro experiments pertain to the in vivo situation as well. To a large extent, however, such an assumption has underpinned the proposition that 5-HT plays a principal role in the development of CVS, for it was argued that its ED$_{50}$ value of $6.3 \times 10^{-9}$ M (as initially reported for dog basilar artery in vitro) was well within physiological concentrations.$^{19}$ The fact that subsequent investigators have generally found higher concentrations for the ED$_{50}$ of 5-HT, ranging from $4.3 \times 10^{-8}$ M to $5 \times 10^{-7}$ M,$^{14,20-22}$ is perhaps less salient than the nearly 1000-fold higher ED$_{50}$ concentration obtained with the in vivo preparation. The likelihood of major differences between in vivo and in vitro determinations of dose-response values was suggested by Boisvert et al$^{23}$ and could result from a number of factors, including the direct accessibility of vasoactive agents to both lumen and cut ends of vessels studied in vitro.

Denervation Supersensitivity

In the strict sense, supersensitivity refers to a state in which tissue shows a greater-than-normal response to a given concentration of agent (with or without change in maximal response), a state that implies alteration of the postjunctional membrane and/or intracellular mechanisms.$^{24}$ Denervation is only one of several causes of this state and has most commonly been associated with striated muscle, presumably because an underlying mechanism of receptor spread beyond the endplate has been elucidated. It is interesting to note, however, that the phenomenon of supersensitivity was first recognized in the denervated dilator muscle of the pupil and has subsequently been observed in a wide range of smooth muscle tissues.$^{24}$

Supersensitivity of smooth muscle has several distinguishing features, such as nonspecificity and considerably lower magnitude in comparison with that of striated muscle. Thus, for most vascular smooth muscle systems that have been studied, the increase in sensitivity to agonists has generally ranged from nil to fourfold, whereas the increase in sensitivity of denervated striated muscle to acetylcholine approaches a factor of 1,000.$^{24}$ The mechanisms underlying denervation supersensitivity in smooth muscle remain to be elucidated, but increasing evidence points toward alterations in the rate of sodium-potassium exchange.$^{25}$

Confirmation that denervation supersensitivity plays a role in the development of SAH-induced CVS would require an affirmative answer to each of the following questions: 1) Does SAH produce denervation of cerebral vessels? 2) Does that denervation induce supersensitivity to a degree that could account for the severity of arterial narrowing associated with SAH? 3) Are vasoconstrictor agents, even under conditions of supersensitivity, present in sufficient concentrations to cause excessive cerebral vasoconstriction?

1. With regard to the question of SAH-induced denervation, our laboratory has recently provided ultrastructural evidence that vessels encased in clotted blood suffer widespread damage to neural elements. Of the time periods examined, the degree of injury was
greatest at 7 days after experimental SAH, both dense-and clear-core varicosities and myelinated as well as unmyelinated axons were affected. The time course of damage to neural elements closely paralleled that to endothelium and smooth muscle, suggesting that all layers of the vascular wall may be injured by common mechanisms. It would appear, therefore, that SAH can produce a profound denervation of cerebral arteries.

2. There has been considerable variability in the results of previous studies on denervation supersensitivity in cerebral vessels. In cases where heightened responsiveness has been found after sympathetic denervation, the magnitude of shift in ED₉₀ values has been similar to that of other vascular systems, generally two- to threefold. On the other hand, several studies in addition to the present report have detected no change in the response of sympathetically denervated cerebral arteries to adrenergic agents, fresh blood, or incubated blood. Toda and coworkers, using contraction chamber measurements of vessels previously subjected to experimental SAH, likewise observed no increase in sensitivity to K⁺, 5-HT, fresh blood, or incubated blood. Remarkably, Kodama et al reported that sympathectomy not only failed to induce supersensitivity, but significantly diminished the contraction of cerebral vessels, prompting their subsequent clinical use of cervical sympathectomy as preventive therapy for CVS. One possible objection to the present study, as well as to previous investigations on denervation supersensitivity of cerebral vessels, that only the sympathetic supply was interrupted and that other neural input, if removed, could lead to a supersensitivity that was overlooked. Similarly, one might point out that not all possible vasoconstrictive agents were tested nor were time periods other than 7 days postdenervation examined. The first response to these concerns is to emphasize that, although an increasing number of neurotransmitters and other vasoactive agents have been detected in cerebral vessels, the chief vasoconstrictor released from neural elements is still considered to be norepinephrine. Second, as mentioned above, denervation supersensitivity in smooth muscle tissue is relatively nonspecific, and the absence of heightened response to norepinephrine, epinephrine, and 5-HT, as well as fresh or incubated blood, minimizes the likelihood of a remaining substance to which the artery was uniquely supersensitive. Another feature of smooth muscle supersensitivity is its relatively slow but tissue-specific time course for development. Previous studies of vascular smooth muscle have found that supersensitivity occurs within 24 hours and reaches a maximum level after 3 days. Since the present investigation focused on the period when both experimental and clinical forms of vasospasm are most pronounced, the onset of a heightened response at later periods cannot be ruled out. On the other hand, the relevance of such supersensitivity to SAH-induced CVS would be minimal.

Finally, it should be mentioned that although in vivo measurements of dose–response properties of arteries may be somewhat less precise than in vitro determinations, the former seem more appropriate for assessing the ultimate physiological consequences of altered reactivity. We believe, therefore, that while the present study does not categorically dismiss the possibility of subtle changes in responses of cerebral arteries deprived of their sympathetic supply, it indicates that any such changes are well below the magnitude required for CVS.

3. Although the levels of 5-HT, norepinephrine, and epinephrine in incubated blood might not be exactly those in subarachnoid blood, the measured values directly address arguments that have been used to support the role of these agents in CVS. The proposal that 5-HT is a major contributor to CVS, for example, continues to be based on the premise that this agent is progressively released from platelets in clotted blood. In the absence of any obvious reason for a significantly different pattern of 5-HT release from clotted blood in the subarachnoid space, it is interesting to note that 5-HT levels of blood maintained in a closed chamber uninterruptedly decrease over time. In fact, the case can be made that the levels of 5-HT and catecholamines in subarachnoid blood would diminish even more rapidly due to diffusion into the pool of circulating cerebrospinal fluid (CSF). Moreover, there is considerable direct and indirect evidence that 5-HT
FIGURE 3. Electron micrographs showing classical degeneration of a dense core varicosity (top) and of axons (bottom) in cat basilar artery 7 days after resection of the superior cervical ganglia.
and monoamine metabolites exist in minimal concentrations in CSF at the time CVS becomes most pronounced. In essence, several lines of evidence now indicate that neither 5-HT nor catecholamines accumulate in sufficient amounts to account for the profound decrease in arterial caliber associated with CVS.

The direction of change in hemoglobin concentration in incubated blood is nearly opposite that of 5-HT. This difference is perhaps partially explained by the considerably longer in vitro half-life of red blood cells compared with platelets. In addition, it appears likely that the degradation of free 5-HT is more rapid than that of hemoglobin. Substantial literature linking hemoglobin and CVS has now accrued, and the amounts of hemoglobin present in incubated blood adds to the reasons for investigating this relationship. Although much of the work on hemoglobin has centered on its vasoconstrictive properties or release of free radicals during catabolism, recent experiments by Furchgott et al have shown that hemoglobin is also capable of inhibiting an endothelial-derived vasodilator.

In summary: The results of this study indicate that adrenergic denervation of cerebral arteries does not significantly alter their sensitivity to vasoconstrictive agents. To the extent that information from cat cerebral vessels applies to human arteries, the findings further suggest that 5-HT, norepinephrine, and epinephrine do not play direct roles in the development of CVS, whereas hemoglobin and its metabolites remain among the strong contenders.

References

KEY WORDS • denervation • cerebral vasospasm • serotonin • hemoglobin • catecholamines
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