EFFECT OF STA-MCA ANASTOMOSIS ON MCA OCCLUSION/Levinthal et al. 375


Role of Adrenergic Nerves in Blood-Induced Cerebral Vasospasm
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SUMMARY Cerebral arteries have an abundant supply of adrenergic nerve fibers which are believed to release vasoactive substances responsible for the induction of cerebral vasospasm. To assess the importance of adrenergic nerves in this phenomenon, high doses (600 μg/ml) of 6-hydroxydopamine (6-OHDA) were used to produce in vitro chemical sympathectomy in bovine middle cerebral artery. 6-OHDA reduced catecholamine fluorescence to undetectable limits. H3-norepinephrine re-uptake was reduced to 1.5% of intact controls. Arterial norepinephrine content was reduced by 92%.

Contractile responses to norepinephrine, serotonin, and fresh human whole blood were modestly reduced after denervation. This reduction was probably due to alpha receptor inactivation by 6-OHDA, because after protection of the alpha receptors with phentolamine the vessel response was the same as in untreated controls. Contractions in response to aged human whole blood were not affected by denervation. The results suggest that the endogenous release of catecholamines does not play a major role in the initiation or spread of blood-induced vasospasm in large cerebral arteries.

THE PHENOMENON of cerebral vasospasm following aneurysm rupture and the clinical problems associated with it are well documented.4 (Study of the responses of bovine middle cerebral vessel has been made in vitro to define some of the underlying mechanisms of the phenomenon of vasospasm in previous work from this laboratory.)4 The results implicate serotonin (5-HT), released from whole blood, as a primary agent responsible for the initiation of early (first phase) vessel contraction. Studies reported recently indicated that breakdown products of erythrocytes in aged whole blood are important factors in prolongation of spasm and the initiation of delayed spasm (second phase). An event not readily explicable by a vasoactive agent released in whole blood is the occurrence of diffuse spasm remote from the local accumulation of blood. One explanation is that the adrenergic innervation of these larger cerebral vessels plays a role in the pathogenesis and spread of spasm, that a spasmogenic agent or agents trigger release of the catecholamine (CA) norepinephrine (NE) from adrenergic nerves, or that the adrenergic nerves may spontaneously release NE which initiates the spasm. The presence of adrenergic nerve terminals in the cerebral artery of many species is well documented.5–11 By using 6-hydroxydopamine (6-OHDA), a substance known to produce functional...
adrenergic denervation in vitro, an attempt was made to assess the importance of endogenous release of NE in the initiation and spread of cerebral vasospasm.

Methods

Bovine middle cerebral arteries were obtained from a local slaughterhouse immediately after sacrifice, cleaned and cut in a helical manner. The tissue was maintained at 37°C and oxygenated continuously in a physiologic Krebs solution of the following mM composition: NaCl 118.9; KCl 4.7; KH₂PO₄ 1.2; CaCl₂ 1.2; MgSO₄·7H₂O 1.2; NaHCO₃ 14.9; dextrose 5.6. pH was maintained at 7.4 and aerated with 12% O₂, 5% CO₂. Osmolarity of the solution was 276 milliosmoles. Arterial contractions were measured isotonically in a manner previously reported and expressed as mm/cm resting length of strip (RLₒ). Two hours were allowed for equilibration. Viability of the tissue was determined by the rate of adjustment of the strips to resting length upon the application of tension (1.25 gm). Strips that did not stretch at a rate of 0.5 mm/min were rejected. When fresh blood-induced contractions were studied, human whole blood (0.1 ml) was added immediately upon withdrawal to the tissue bath. To produce a contraction with aged blood, whole blood was kept aseptically at 37°C in an incubator for 7 days, and 0.1 ml was added to the 10 ml tissue bath. In both cases blood was left in contact with the muscle strip for 12 hours.

Denervation

6-hydroxydopamine (300 or 600 µg/ml of the HBr salt) was used to produce destruction of adrenergic nerve terminals according to the method of Aprigliano. Since 6-OHDA is quickly oxidized at high pH, a low pH vehicle solution containing the reducing agent glutathione was used to reduce the rate of oxidation. The vehicle had the following composition in mM: NaCl 137; MgCl₂ 1.2; KCl 2.7; CaCl₂ 1.8; dextrose 7.8; EDTA 0.03. Glutathione (30 µM) was added to the unbuffered vehicle and the pH was adjusted to 4.9 with NaOH. The tissue was transferred to the low pH solution before the addition of 6-OHDA. After a 10 min exposure the strips were returned to the N-Krebs solution and incubated 2 hours to allow denervation to complete. Control tissues were handled in an identical manner except for the addition of 6-OHDA. No difference was noted in viability between control and 6-OHDA-treated tissue groups before and after 6-OHDA were evaluated by Student's t-test for unpaired samples.

Catecholamine Determinations

The efficacy of 6-OHDA destruction of noradrenergic nerve terminals was determined by measuring both the concentration of NE in the vessel and the viability of vesicular re-uptake mechanisms. NE concentrations were quantified histochemically and radioenzymatically (REM). Active uptake of NE (Uptake 1) was measured using 1-NE-7-H (New England Nuclear (0.1 Ci/mM). The uptake studies were made after 6-OHDA treatment by incubating tissue for one hour in N-Krebs medium containing 9.5 ng/nl HNE + 20 µg/ml ascorbic acid, then frozen in 2-methylbutane in liquid N₂. Control tissues were treated identically with the exception of 6-OHDA addition. Tissues for REM and NE uptake measurements were homogenized in 2 ml 0.4N perchloric acid. One hundred µl aliquots were taken for duplicate REM determinations, and the remainder was buffered to pH 7.8, placed on alumina, and CA removed with 3 ml 0.05PCA. NE was then isolated on a Dowex 50xW ion exchange resin. REM from this fraction was evaluated by scintillation spectrophotometry. Adjacent tissue was processed for fluorescence viewing of CA by the standard Falck-Hillarp condensation technique.

Drugs

All drugs were dissolved in double deionized water and added directly to the bath, some in a cumulative manner to produce a dose response curve. Concentrations are expressed as the final molar concentration present in the bath. Phentolamine mesylate, 75 µg/ml (Ciba), was added 10 min before 6-OHDA HBr (Sigma), then washed out with the 6-OHDA 10 min later. Previous experiments have shown maximum inhibition of both NE and 5-HT contractions with this dose of phentolamine in bovine middle cerebral artery (unpublished observations). A specific uptake blocker, Desmethylimipramine HCl (DMI) was used in some instances to expose extrasynaptic (Uptake 2) contamination of uptake measurement. DMI (SKF) was added 10 min before 1H-NE and left in the bath during uptake. Calcium-free high potassium Krebs solution was made by increasing the potassium to 70 mM and eliminating all calcium. Osmolarity was adjusted to 275 by decreasing NaCl. After washing the tissue with 70 mM K⁺, Ca-free solution 3 times, CaCl₂ was added in a cumulative manner. Also used were 1-noradrenaline bitartrate (Sigma) and serotonin HCl (Sigma). Mean ± SE of contractile responses for all groups are reported. Significant differences between groups before and after 6-OHDA were evaluated by the Student's t-test for unpaired samples.

Results

6-OHDA has been shown to produce selective destruction of adrenergic nerve terminals in vitro, as well as in vivo. With 300 µg/ml of this toxin, Aprigliano was able to completely denervate the caudal artery of the rat. However, in the present study when bovine middle cerebral arteries were exposed to this dose, CA varicosities, while greatly reduced, were still present when visualized histochemically. The reduction in CA fluorescence generally paralleled the decline in NE uptake efficiency, which was reduced 52.5 ± 0.6% (p < 0.005) with respect to controls (fig. 1). Contractions produced by serotonin, NE or human whole blood were not changed after exposure to 300 µg/ml 6-OHDA as compared to control.
TABLE Tissue Content of Norepinephrine in Bovine Cerebral Artery Before and After 6-Hydroxydopamine

<table>
<thead>
<tr>
<th>N</th>
<th>µg/g wet wt.</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.31 ± 0.09</td>
</tr>
<tr>
<td>300 µg/ml</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td>600 µg/ml</td>
<td>0.10 ± 0.01</td>
</tr>
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When the 6-OHDA concentration was increased to 600 µg/ml, CA fluorescence was no longer detectable (fig. 2a-b). Uptake of HNE was reduced to 15.3 ± 0.3% of intact controls (fig. 1). To determine if this remaining uptake of HNE was specific vesicular (Uptake 1) or non-specific extravesicular (Uptake 2), DMI (10⁻⁷ M) was used to block neural uptake sites before the addition of HNE, but after treatment with 600 µg/ml 6-OHDA uptake values were similar to tissue treated with 6-OHDA without DMI, 14.3 ± 15% (fig. 1). Thus, uptake of NE at this dose of 6-OHDA, when corrected for non-specific binding, was reduced to 1.5% or less of control.

When the NE content of the vessels was determined radioenzymatically, the same general pattern was observed. NE arterial concentration was reduced by 26% after 300 µg/ml 6-OHDA, while 600 µg/ml 6-OHDA reduced NE by 92% relative to controls (table). The remaining 8% is probably NE sequestered extravesicularly.

Dose response curves produced by 5-HT after 600 µg/ml 6-OHDA were significantly depressed by an average of 30% throughout the dose response range (p < 0.025) (fig. 3). NE responses were also significantly depressed, more so at higher doses (10⁻⁷ to 10⁻⁴ M) (fig. 4). This inhibition of the contractile response could be the result of inactivation or damage of the smooth muscle alpha receptors. Thus, the receptors were protected with 75 µg/ml of phentolamine prior to the addition of 6-OHDA. After protection of the alpha receptors, 5-HT responses continued to be suppressed significantly (34%; p < 0.025) at the lower end of the curve, but contractions approached control responses at the higher end of the curve (fig. 3). NE responses did not differ significantly from controls after phentolamine (fig. 5). Control experiments indicated no residual effects with phentolamine after 3 washings.

FIGURE 2. A) Fluorescence photograph of bovine middle cerebral artery. Tangential section through the middle cerebral artery showing the web-like network of brightly fluorescent nerve fibers surrounding the vessel. (100 ×) B) Cross section of an artery treated with 600 µg/ml 6-hydroxydopamine for 2 hours. Fluorescence is completely absent. Only autofluorescence is present. (100 ×)
High concentrations of 6-OHDA could also cause non-specific depressant action on smooth muscle, thereby reducing the contractile effect of the agonists. Impaired movement of calcium across a depolarized membrane should be an indication of such damage. Figure 6 demonstrates that this is not the case. Little difference is noted in the calcium contractile curve after 6-OHDA.

With NE uptake effectively curtailed in the absence of apparent muscle cell damage, the effect of 6-OHDA on human whole blood contractions was examined. Arterial strips were exposed to 0.1 ml of either fresh or aged human whole blood. The amount of peak contraction and time to return to baseline were recorded.

Figure 3. Effect of 6-OHDA (6-hydroxydopamine, 600 μg/ml) on serotonin dose response contractions before and after protection of alpha receptors with phentolamine (75 μg/ml). Closed square, open square — mean ± se of 17 strips, asterisk indicates significant difference, (p < 0.025) between control and 6-OHDA treated tissue, stars — mean ±SE of 8 strips, circle indicates significant difference (p < 0.025) between control and 6-OHDA + phentolamine treated tissue.

Figure 4. Effect of 6-OHDA (6-hydroxydopamine, 600 μg/ml) on norepinephrine dose response contraction. Mean ±se of 16 strips. *p < 0.005, **p < 0.05.

Figure 5. Effect of 6-OHDA (6-hydroxydopamine, 600 μg/ml), after protection of alpha receptors with phentolamine (75 μg/ml), on norepinephrine dose response contraction. Mean ± se of 8 strips.

Figure 6. Calcium dose response curve in the absence and presence of 6-OHDA (6-hydroxydopamine, 600 μg/ml). Mean ±se of 9 strips.
Fresh whole blood contractions were significantly reduced (p < 0.025) after 600 µg/ml (control 0.688 ± 0.20 mm/cm RL0; treated 0.460 ± 0.05 mm/cm RL0 (fig. 7). Time to return to baseline was not significantly changed (control 9.0 ± 1.4 hr; treated 6.2 ± 1.3 hr). Contractile responses to aged whole blood did not differ significantly after 6-OHDA treatment (control 1.28 ± 0.14 mm/cm RL0; treated 1.03 ± 0.26 mm/cm RL0). Time to return to baseline also remained unchanged (control 7.5 ± 0.6 hr; treated 7.7 ± 1.0 hr).

Discussion

Although adrenergic nerves are present in sufficient numbers to play an active role in overall regulation of cerebral vascular tone,\(^7\) there is controversy concerning their function. It is unclear whether endogenous release of catecholamines is a major factor in the initiation or spread of blood-induced vasospasm in large cerebral arteries.

6-OHDA acutely and chronically depletes postganglionic sympathetic nerves of NE stores \(in\) \(vivo\)\(^8\) and \(in\) \(vitro\). In this study high concentrations (600 µg/ml) of 6-OHDA were required to deplete a bovine cerebral artery. Rosenblum\(^9\) had similar difficulty in depleting rat cerebral artery of NE with reserpine and suggested that cerebral vessels bind NE more than nerves to vessels outside the brain.

Evidence of adrenergic impairment following 6-OHDA administration was obtained by the loss of CA fluorescence and the highly significant reduction in \(^3\)HNE uptake. Subsequent contractions with serotonin and fresh blood were reduced, but it was not determined whether the reduction was due to loss of releasable endogenous CA, or smooth muscle receptor damage, or both. High doses of 6-OHDA can block or destroy alpha adrenergic receptor sites.\(^10\) The depressed NE contractions after 6-OHDA were apparent due to this action as protection of the alpha receptors with phentolamine returned contractile responses to control level.

The higher end of the 5-HT dose response curve was not significantly depressed after phentolamine, while the low end of the curve continued to be reduced suggesting the existence of specific 5-HT receptors that may be affected by 6-OHDA. Such receptors, specific for 5-HT, have recently been described in the cat.\(^21\) It may be that when 5-HT receptors become occupied, 5-HT may act via the alpha receptors, or high concentrations of 5-HT may release NE from nerve terminals. Based on a study using dog saphenous vein, Humphrey\(^22\) suggested that low concentrations of serotonin act directly on specific serotonin receptors while high concentrations can act indirectly on adrenergic nerve terminals by displacing norepinephrine from neuronal stores. It is known that 5-HT can stimulate NE release from the sympathetic nerve of the rabbit heart by activating tryptamine receptors.\(^23\) Similar results have been obtained with guinea pig vas deferens\(^24\) and cat spleen capsules.\(^25\) However, recent work by McGrath\(^26\) in canine saphenous vein and tibial artery indicates that 5-HT can depress sympathetic tone by inhibiting the release of adrenergic transmitters. Phentolamine did not have a residual effect, since control experiments without 6-OHDA showed that complete washout of phentolamine could be achieved after similar treatment procedures.

Responses of arteries to calcium in a high potassium solution were unaffected after 6-OHDA, suggesting that high doses of 6-OHDA were not depressing or destroying the smooth muscle cells.

Shibata\(^27\) has shown that fluorescence in adrenergic fibers is not always a reliable index of 6-OHDA depletion of NE, so \(^3\)HNE uptake and content were also measured. CA uptake and retention were effectively curtailed as evidenced by the drastic reduction in the \(^3\)HNE concentrations. After the addition of DMI, which inactivates the monoamine Uptake 1 site,\(^28\) uptake values remained the same. Thus, nearly 100% of the vesicular NE uptake was inhibited. Loss of at least sympathetic function, however, is not dependent upon the total depletion of NE stores. Goldberg and Kadowitz,\(^29\) using dog saphenous vein, showed that \(in\) \(vitro\) application of 6-OHDA (25 µg/ml) will cause functional denervation as indicated by loss of contractions after transmural nerve stimulation. The effects were reproducible, blocked by DMI, and were not found to be transient.

Fresh whole blood contraction, corresponding to early spasm, was reduced significantly after 6-OHDA. The reduction was comparable to that seen at the lower end of the 5-HT dose response curve after similar treatment. The majority of the contractile substance in fresh human whole blood has been postulated to be 5-HT.\(^3\) Garattini\(^30\) found 5-HT levels about 10^4 M in human whole blood. This suggests that the reduction of contractions after fresh whole blood can be attributed to the direct effect of 6-OHDA on 5-HT receptors. Experiments using methysergide to
protect the 5-HT receptor sites were unclear, since methysergide had residual effects even after repeated washings. The ability to maintain a contraction after application of human whole blood, indicated by the time to return to baseline, was unchanged after 6-OHDA.

Recent research on vasospasm has indicated that a vasoactive substance appears in incubated whole blood after 2 to 7 days. This substance has been tentatively identified as an oxyhemoglobin derivative. The contractions after application of aged blood, which may be comparable to late spasm, were unchanged after denervation and the relaxation rate did not differ. Contractile responses to aged blood were significantly greater than to fresh blood indicating an increase in vasoactive substance present in old blood or, more probably, the production of a new contractile substance. It is unlikely that serotonin or norepinephrine remain in old blood after 7 days of incubation because of their continuing oxidation.

Nerve severance due to the in vitro technique used may have eliminated CA adrenergic feedback mechanisms normally operative in vivo.

Although NE may play a contributing role in vasospasm, the data from this study suggest that spontaneous release of NE from adrenergic nerves is probably not the primary factor responsible for the pathogenesis of blood-induced spasm.

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