Changes of neuropeptide immunoreactivity in cerebrovascular nerve fibers after experimentally produced SAH

Immunohistochemical study in the dog

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The immunoreactivity of vasoactive intestinal polypeptide (VIP)-, substance P (SP)-, and neuropeptide Y (NPY)-containing nerve fibers in the basilar artery (BA) and proximal portion of the middle cerebral artery (M1) was immunohistochemically examined in the dog after experimentally produced subarachnoid hemorrhage (SAH). The SAH was produced by a single injection of fresh autologous arterial blood (1 ml/kg body weight) into the cisterna magna. The density (the averaged number of nerve fibers in a unit area) of VIP-, SP-, and NPY-immunoreactive perivascular nerve fibers in the M1 segment and the BA was markedly decreased (5% to 40% of the normal value) immediately after the injection. The density of VIP- and SP-immunoreactive perivascular fibers increased 2 or 3 weeks after SAH and became normal by the 63rd day after injection. On the other hand, no substantial recovery was observed in the density of NPY-immunoreactive perivascular fibers by 63 days after injection.

KEY WORDS • subarachnoid hemorrhage • vasoactive intestinal polypeptide • substance P • neuropeptide Y • cerebral vasculature • dog

Cerebral vasospasm is a major complication of subarachnoid hemorrhage (SAH), and its pathogenesis is still a matter of debate. Some authors have emphasized neurogenic factors, suggesting that catecholaminergic perivascular nerve fibers of cerebral arteries may play a role in the genesis of cerebral vasospasm. In the last decade, immunohistochemical studies have demonstrated that cerebral arteries are accompanied by many perivascular nerve fibers containing vasoactive neuropeptides, such as vasoactive intestinal polypeptide (VIP), substance P (SP), and neuropeptide Y (NPY). Cell bodies of these nerve fibers appear to be located in the sphenopalatine, trigeminal, and superior cervical ganglia. Neuropeptide Y has been reported to show a potent vasoconstrictive action on cerebral arteries in vivo as well as in vitro, and to coexist in part with catecholamines in sympathetic ganglion cells and postganglionic fibers. On the other hand, VIP causes vasodilation of cerebral arteries. Substance P also exerts relaxation effects, although not as potent as those of VIP, on cerebral arteries in situ and in vitro. Thus, perivascular nerve fibers containing these vasoactive neuropeptides may possibly exert vasomotor actions upon cerebral arteries.

In the present study, a model of SAH was produced in the dog, and then the VIP-, SP-, and NPY-like immunoreactivity in the perivascular nerve fibers of the main cerebral arteries was examined immunohistochemically during the post-SAH period.

Materials and Methods

Mongrel dogs of either sex, ranging in weight from 4.0 to 8.0 kg, were used in this investigation. In 21 dogs, SAH was produced by injecting autologous blood into the cisterna magna as follows. The dogs were anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg body weight), after which endotracheal intubation...
and artificial ventilation with room air were instituted. Fresh autologous blood was obtained from the femoral artery, and introduced under sterile conditions in a single injection (1 ml blood/kg body weight) into the cisterna magna. Following injection, the dogs were tilted in the head-down position for 30 minutes to facilitate settling of the blood by gravity into the basal cisterns. At the completion of the operation the dogs were treated with antibiotic agents and were kept in sanitary conditions for varying lengths of time until sacrifice. Three dogs each were killed on post-SAH Days 1, 3, 7, 14, 21, 42, and 63. At the time of sacrifice, the dogs were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with 1 liter of 0.9% saline, followed by a fixative (1 liter/kg body weight) containing 1% picric acid and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3).

In three other dogs a sham operation was performed. The dogs were treated in the same way as those undergoing SAH except that they received a single injection of 0.9% saline (1 ml/kg body weight) into the cisterna magna and were allowed to survive for 7 days before sacrifice by cardiac perfusion. Additionally, three normal dogs were subjected to transcardiac perfusion only as described above.

After perfusion, the large pial arteries as well as the superior cervical, trigeminal, and sphenopalatine ganglia were removed, washed, and immersed in 0.1 M phosphate-buffered 0.9% saline (PBS, pH 7.3). The basilar artery (BA) and proximal portion of the middle cerebral artery (M,) were cut transversely into three segments (see Fig. 1). These segments were soaked for 30 minutes in PBS containing 2% non-immune goat serum and were then exposed to well-characterized rabbit antisera against VIP diluted 1/1500, against SP diluted 1/6000, or against NPY diluted 1/3000 for 24 hours at 4°C.* Subsequently, biotinylated goat anti-rabbit immunoglobulin G antibody and avidin-biotinylated peroxidase complex were conjugated to each primary antibody at room temperature over a period of 24 hours for each incubation.† Immunolabeled peroxidase was visualized by incubation at room temperature for 5 to 10 minutes with 0.015% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6). The ganglia were saturated with the same PBS containing 20% sucrose and were then cut into sections 50 μm thick on a freezing microtome. The sections were rinsed with several changes of the same PBS and immunostained in the same manner as the segments of M, and BA.

Preincubation of the diluted antiserum against VIP, SP, or NPY, with a 50-μg/ml concentration of VIP, SP, or NPY, respectively, resulted in elimination of all immunohistochemical staining. Omission of antibody at each incubation step also produced negative staining.

The density of immunoreactive perivascular nerve fibers in the M, segment and the BA was estimated by an averaged number of the fibers in a unit area 0.2 x 0.2 sq mm in size; in each dog, five unit areas were sampled randomly in each M, segment and each BA, and the numbers of immunoreactive fibers in these areas were counted, as indicated in Fig. 2, and then averaged. Comparison of the density (the averaged number of immunoreactive perivascular nerve fibers in each 0.04-sq mm area) was made using the unpaired two-tailed Student t-test; a difference in density was considered to be significant when p was less than 0.02.

**Results**

In all the dogs that were injected with autologous arterial blood into the cisterna magna and allowed to survive for 1, 3, or 7 days, SAH was confirmed by gross inspection at the time of sacrifice. In these animals, subarachnoid clot and/or xanthochromic staining of Vasoactive intestinal polypeptide and substance P obtained from Protein Research Foundation, Osaka, Japan; neuropeptide Y obtained from Sigma Chemical Co., St. Louis, Missouri.

* Rabbit antiserum against vasoactive intestinal polypeptide obtained from Immunonuclear Corp., Stillwater, Minnesota; rabbit antiserum against neuropeptide Y obtained from Amersham Corp., Buckinghamshire, England.
† Biotinylated goat anti-rabbit immunoglobulin G antibody and avidin-biotinylated peroxidase complex obtained from Vector Laboratories, Inc., Burlingame, California.

† Vasoactive intestinal polypeptide and substance P obtained from Protein Research Foundation, Osaka, Japan; neuropeptide Y obtained from Sigma Chemical Co., St. Louis, Missouri.
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FIG. 2. Photomicrographs of vasoactive intestinal polypeptide (VIP)-immunoreactive perivascular nerve fibers in unit areas of the proximal portion of the middle cerebral artery (M1) in a normal dog (a) and in an operated dog on the 7th day after subarachnoid hemorrhage (SAH) (b). Whole mounts, × 235. The corresponding schematic orientation of nine (a) and four (b) immunoreactive nerve fibers counted in the unit area is shown to the right of each photomicrograph.

In the normal dogs, many VIP-, SP-, and NPY-immunoreactive perivascular nerve fibers were seen in the BA and in the M1 segment (Figs. 2a, 3a, 4a, and 5a). The density of the VIP- and NPY-immunoreactive nerve fibers was larger in the M1 portion than in the BA; the mean density (± standard error of the mean) of VIP was 7.7 ± 0.48/0.04 sq mm in the M1 segment and 3.8 ± 0.30/0.04 sq mm in the BA, and that of NPY was 7.8 ± 0.10/0.04 sq mm in the M1 segment and 6.6 ± 0.27/0.04 sq mm in the BA. On the other hand, SP-immunoreactive nerve fibers in the M1 segment were less numerous than VIP- and NPY-immunoreactive fibers; the mean density of the SP-immunoreactive fibers was 3.2 ± 0.10/0.04 sq mm in the M1 segment and 4.1 ± 0.22/0.04 sq mm in the BA (Table 1).

Immunoreactivity to VIP, SP, or NPY was also seen in cell bodies of ganglion cells in the sphenopalatine, trigeminal, or superior cervical ganglion, respectively (Fig. 6). Most of the sphenopalatine ganglion cells showed VIP-like immunoreactivity. An SP-like immunoreactivity was seen in most small neurons in the trigeminal ganglion. Many neurons in the superior cervical ganglion showed NPY-like immunoreactivity.

In the sham-operated dogs, the VIP-, SP-, and NPY-like immunoreactivity in the cerebral arteries and ganglion cell bodies was substantially the same as that in the normal dogs (Fig. 7 and Table 1).

In the dogs with SAH, VIP-, SP-, and NPY-like immunoreactivity in the M1 segment and the BA was markedly reduced in the acute period after production of the SAH (Fig. 7 and Table 1). The density of the...
FIG. 4. Photomicrographs of substance P-immunostained middle cerebral arteries taken from a normal dog (a) and from a dog with induced subarachnoid hemorrhage (SAH) that was allowed to survive for 14 days after the SAH (b). Whole mounts, $\times 150$.

FIG. 5. Photomicrographs of neuropeptide Y-immunostained middle cerebral arteries taken from a normal dog (a) and from a dog with induced subarachnoid hemorrhage (SAH) that was allowed to survive for 42 days after the SAH (b). Whole mounts, $\times 150$.

immunoreactive perivascular nerve fibers in the $M_1$ segment and the BA was 5% to 40% of control values 24 hours after SAH, and remained less than 60% of control values during the 1st week after SAH. A few fine varicose fibers showing VIP- and SP-like immunoreactivity were seen in the $M_1$ segment and the BA on the 7th and 14th days after SAH (Figs. 2b, 3b, and 4b). The reduction was most prominent in the VIP-immunoreactive nerve fibers. However, the density of the VIP- and SP-immunoreactive nerve fibers began to recover by 2 or 3 weeks after SAH. Fine varicose fibers showing VIP-like immunoreactivity again became apparent in the $M_1$ segment and the BA on the 21st day after SAH (Fig. 3c). The density of VIP- and SP-immunoreactive nerve fibers in both the $M_1$ segment and the BA was in the normal range by the 63rd day after SAH (Figs. 7a and b and Table 1). On the other hand, no clear tendency to recover was observed in the density of NPY-immunoreactive perivascular nerve fibers in the $M_1$ segment and the BA on the 42nd day after SAH (Fig. 5b), and the density of NPY-immunoreactive perivascular nerve fibers in these vessels remained less than 50% of the control value, even on the 63rd day after SAH (Fig. 7c). In the sphenopalatine, trigeminal, and superior cervical ganglia, no changes were found in VIP-, SP-, and NPY-like immunoreactivity in the post-SAH period.

**Discussion**

In the present study, an SAH model was produced in the dog by a single injection of fresh autologous blood into the cisterna magna, and the immunoreactivity of VIP-, SP-, and NPY-containing perivascular nerve fibers in the $M_1$ arterial segment and the BA was subsequently examined in the post-SAH period. In the normal and sham-operated dogs, VIP- and NPY-immunoreactive perivascular nerve fibers were seen more abundantly in the $M_1$ segment than in the BA. Similar findings concerning the distribution of VIP- and NPY-like immunoreactivity in large pial arteries have been reported in the rat$^{1,20}$ and in man.$^{2,6}$ On the other hand, we found no substantial differences in the density of SP-immunoreactive nerve fibers between the $M_1$ segment and the BA, as have been reported in the cat.$^{22}$

In the dogs in which SAH was produced, the density of VIP-, SP-, and NPY-immunoreactive perivascular nerve fibers in the $M_1$ segment and the BA was mark-
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**FIG. 6.** Photomicrographs of sections through the sphenopalatine (a), trigeminal (b), and superior cervical (c) ganglia in normal dogs. The sections were immunostained respectively for vasoactive intestinal polypeptide (a, × 240), substance P (b, × 150), and neuropeptide Y (c, × 240).

**FIG. 7.** Graphs showing percent changes in the mean values of the density of vasoactive intestinal polypeptide- (VIP, upper left), substance P- (SP, upper right), and neuropeptide Y- (NPY, lower) immunoreactive perivascular nerve fibers in the proximal portion of the middle cerebral artery (M1, open circles) and in the basilar artery (BA, filled circles) 1 to 63 days after subarachnoid hemorrhage. The mean density of the immunoreactive fibers in the sham-operated dogs is also shown for the M1 segment (open triangles) and for the BA (filled triangles). The vertical bars indicate 2 standard errors.
edly decreased in the initial post-SAH period. Although the density of VIP- and SP-immunoreactive perivascular nerve fibers gradually recovered to normal values, that of NPY-immunoreactive perivascular nerve fibers was still suppressed on the 63rd day after SAH. The decrease in immunoreactive perivascular nerve fibers at the initial stage after SAH might be ascribable to: 1) degenerative loss of immunoreactive neurons; 2) arrest of axonal transport of the neuropeptides, which might result from local damage to axons at the perivascular sites; 3) decreased production of the neuropeptides in the relevant neurons; and/or 4) excessive release and/or block of re-uptake of the neuropeptides at the perivascular terminal sites. Most, if not all, of the VIP-, SP-, and NPY-immunoreactive perivascular fibers in the M1 segment and the BA appear to be supplied by neurons in the sphenopalatine, trigeminal, and superior cervical ganglia, respectively.11,25 According to Svendgaard, et al.,27 catecholamine-induced fluorescence of perivascular sympathetic nerve fibers in cerebral arteries decreases acutely and then recovers at an early stage of the post-SAH period. In the present study, however, the post-SAH decrease in the density of NPY-immunoreactive perivascular nerve fibers in the M1 segment and the BA did not recover even after 63 days. Thus, the present results indicate that VIP-, SP-, and NPY-like immunoreactivity in perivascular nerve fibers of large pial arteries is suppressed by experimentally produced SAH. Although the VIP- and SP-like immunoreactivity gradually recovered, the NPY-like immunoreactivity remained suppressed. It is still uncertain whether and how these changes of the immunoreactivity of vasoactive neuropeptides in perivascular nerve fibers may be involved in the pathological processes that induce cerebral vasospasm in the post-SAH period.

Acknowledgment

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References


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**TABLE 1**

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>VIP-Immunoreactive M1 Segment</th>
<th>VIP-Immunoreactive BA</th>
<th>SP-Immunoreactive M1 Segment</th>
<th>SP-Immunoreactive BA</th>
<th>NPY-Immunoreactive M1 Segment</th>
<th>NPY-Immunoreactive BA</th>
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<tbody>
<tr>
<td>Normal dogs</td>
<td>7.7 ± 0.48</td>
<td>3.8 ± 0.30</td>
<td>3.2 ± 0.10</td>
<td>4.1 ± 0.22</td>
<td>7.8 ± 0.10</td>
<td>6.6 ± 0.27</td>
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<td>Sham-operated dogs (Day 7)</td>
<td>7.1 ± 0.41</td>
<td>4.0 ± 0.30</td>
<td>2.6 ± 0.15</td>
<td>3.3 ± 0.22</td>
<td>7.6 ± 0.24</td>
<td>6.3 ± 0.24</td>
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<td>Day 1</td>
<td>3.0 ± 0.36†</td>
<td>1.3 ± 0.29†</td>
<td>1.3 ± 0.20†</td>
<td>1.0 ± 0.20†</td>
<td>2.4 ± 0.62†</td>
<td>2.1 ± 1.50†</td>
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<tr>
<td>Day 3</td>
<td>2.1 ± 0.41†</td>
<td>1.3 ± 0.32†</td>
<td>1.3 ± 0.21†</td>
<td>1.8 ± 0.27†</td>
<td>2.2 ± 0.40†</td>
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<td>Day 7</td>
<td>2.5 ± 0.29§</td>
<td>0.6 ± 0.12§</td>
<td>1.8 ± 0.31†</td>
<td>1.8 ± 0.30§</td>
<td>2.8 ± 0.44§</td>
<td>1.9 ± 0.37§</td>
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<td>Day 14</td>
<td>4.5 ± 0.26†</td>
<td>1.6 ± 0.46†</td>
<td>1.4 ± 0.20†</td>
<td>2.6 ± 0.35†</td>
<td>1.8 ± 0.24†</td>
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<td>Day 21</td>
<td>3.8 ± 0.18†</td>
<td>2.4 ± 0.20†</td>
<td>1.6 ± 0.27†</td>
<td>2.3 ± 0.22†</td>
<td>1.6 ± 0.14†</td>
<td>2.5 ± 0.15†</td>
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<td>Day 42</td>
<td>5.3 ± 0.20†</td>
<td>2.9 ± 0.18§</td>
<td>1.7 ± 0.18†</td>
<td>2.2 ± 0.14†</td>
<td>4.4 ± 0.41†</td>
<td>3.5 ± 0.25†</td>
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<td>Day 63</td>
<td>6.9 ± 0.24</td>
<td>4.3 ± 0.29</td>
<td>3.3 ± 0.41</td>
<td>4.2 ± 0.27</td>
<td>3.2 ± 0.24†</td>
<td>2.2 ± 0.33†</td>
</tr>
</tbody>
</table>

* Values are means ± standard error of the means for 15 unit areas, each 0.2 x 0.2 sq mm in size. VIP = vasoactive intestinal polypeptide; SP = substance P; NPY = neuropeptide Y; M1 = proximal portion of the middle cerebral artery; BA = basilar artery; SAH = subarachnoid hemorrhage. Significance of difference in data is presented for the normal dogs compared to the dogs undergoing SAH or sham operation († = p < 0.01; § = p < 0.02), and for the sham-operated dogs versus the SAH dogs on the 7th day after the operation (§ = p < 0.01; || = p < 0.02).
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