Perivascular Innervation of the Mesenteric Artery in Spontaneously Hypertensive Rats

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Perivascular innervation of the mesenteric arteries of 7-week-old and 6-month-old spontaneously hypertensive rats and normotensive Wistar-Kyoto rats was examined. The densities of neuropeptide Y–containing nerve fibers and adrenergic nerve fibers were increased in the distal regions of mesenteric arteries of spontaneously hypertensive rats as compared with findings in Wistar-Kyoto rats. However, the densities of cholinergic nerve fibers, vasoactive intestinal polypeptide–containing, and substance P–containing nerve fibers in the mesenteric arteries of the spontaneously hypertensive rats were unchanged in comparison with findings in the Wistar-Kyoto rats. Thus, not only adrenergic nerve fibers but also neuropeptide Y–containing nerve fibers may play an important role in the development and maintenance of hypertension in spontaneously hypertensive rats. (Hypertension 1989;14:660–665)

The adrenergic nerve and cholinergic nerve are vasomotors; it is generally believed the former acts on vasoconstriction and the latter on vasodilation, thus regulating blood flow. It is assumed that the phenomenon of adrenergic nerve dominance in peripheral arteries of spontaneously hypertensive rats (SHR) plays an important role in the development and maintenance of hypertension.1–4 Ichijima1 reported that, in the small arteries of the jejunum in SHR before the development of hypertension and at the early development stage, there was a higher density of innervation of adrenergic nerves compared with the findings in the Wistar-Kyoto (WKY) rats. Head et al2 reported that at any age SHR had larger adrenaline contents in the mesenteric arteries. It is thus considered that histologically, as well as biochemically, the action of the adrenergic nerve in peripheral arteries could be a factor in the development or maintenance of hypertension in SHR.

On the other hand, the concept of autonomic innervation of blood vessels related to the adrenergic and cholinergic nerves has undergone a rapid change with the advance in research on neuropeptides.5–15 However, the relation between the distribution densities of various newly discovered neuropeptide-containing nerve fibers and the development and maintenance of hypertension in SHR are less well understood.

There are reports that the relative number of perivascular nerve fibers expressed histochemically or immunohistochemically in cerebral arteries showed good agreement with data obtained from radioimmunoassay.6,15 In mesenteric arteries of 7-week-old and 6-month-old SHR, we examined histologically the density of innervation of nerves containing various neuropeptides (i.e., neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), and substance P (SP)) as well as those of adrenergic and cholinergic nerves and compared our findings with those found in the control group of WKY rats.

Materials and Methods

Animals used for the studies were male SHR and normotensive WKY rats, 7 weeks and 6 months of age. Superior mesenteric arteries were obtained and three categories of arteries were used as sampling sites and studied (Figure 1). Systolic blood pressures were measured biweekly by tail-cuff plethysmography.

To observe the catecholamine fluorescence in adrenergic nerve fiber, the Falck-Hillarp method16 was used. The mesenteric arteries were removed unfixed. Whole-mount specimens were stretched on glass slides, air-dried for 5 hours in a desiccator containing P2O5, and subsequently treated with paraformaldehyde vapor at 80°C for 1 hour. The specimens were then mounted in buffered glycerol (1:3) for examination under the fluorescence microscope with BG12 filter activation and 490 μm barrier filter.
To demonstrate in cholinergic nerve fiber the acetylcholinesterase positive nerve fibers, the Karnovsky and Roots method was used. The specificity of cholinergic nerves was confirmed by eliminating pseudocholinesterase activity, by addition of iso-OMPA 10^-4 M solution (Sigma Chemical Co., St. Louis, Missouri) to the incubation medium.

The peroxidase-antiperoxidase immunohistochemical technique was used for immunolocalization of NPY-, VIP-, and SP-containing nerve fibers. The rats were anesthetized and perfused through the heart with ice-cold saline followed by Zamboni's fixative. After perfusion, the mesenteric arteries were rapidly removed and postfixed in the same fixative overnight at 4°C. The arteries were washed several times with 0.1 M phosphate-buffered saline (PBS), followed by 10% and 20% sucrose dissolved in the same PBS for more than 1 hour each time. The specimens were thoroughly washed in the same PBS and were then reacted with normal goat serum (code no. 5000, 1:40, Tago, Inc., Burlingame, California) for 1 hour at room temperature. After they were rinsed, the specimens were reacted with anti-NPY (batch no. R-844604, 1:400, Milab, Malmo, Sweden), anti-VIP (lot no. 8608028, 1:3,000, Incstar, Stillwater, Minnesota), and anti-SP (lot no. 8336022, 1:3,000, Incstar) antibodies for 3 days at 4°C. After they were rinsed, the specimens were reacted with goat antirabbit immunoglobulin G (1:200, Cappel, West Chester, Pennsylvania) for 2 hours at room temperature. After another rinsing, the specimens were reacted with peroxidase antiperoxidase (1:200, Cappel) for 2 hours at room temperature. The specimens were again rinsed and left to react with 0.05% 3,3'-diaminobenzidine (Sigma Chemical Co.) in 0.05 M Tris HCl buffer, pH 7.4, plus 0.01% hydrogen peroxide for 3–6 minutes at room temperature. PBS was used for all the rinsing and antibody dilutions. For the light microscopic study, the specimens were dehydrated, coverslipped, and observed under a light microscope.

To check the specificity of NPY, VIP, and SP immunoreactivity, the following controls were performed: 1) incubation with the serum from nonimmunized rabbit as primary antiserum; 2) incubation omitting the primary antiserum in the first step of each peroxidase-antiperoxidase procedure; and 3) incubation with NPY, VIP, and SP antiserum preabsorbed with synthetic NPY (50 µg/ml diluted antiserum, Bioproducts, Brussels, Belgium), synthetic VIP (50 µg/ml diluted antiserum, Bioproducts), and synthetic SP (50 µg/ml diluted antiserum, Bioproducts). Neuronal structures showing NPY, VIP, and SP immunoreactivity were never detected when the tissues were treated with one of the procedures mentioned above.

To quantify transition of the number of nerve fibers, the densities were expressed as the number of nerve fibers crossing an imaginary 1 cm line drawn longitudinally along the middle of each artery on the photomicrographs, according to the counting method described by Kobayashi et al. Student's t test was used to evaluate differences between SHR and WKY rats. Diameter of the artery was measured after fixation when the mesenteric artery was maximally dilated.

Results

In the SHR, the blood pressure in the 7-week-old rats was 152±8 mm Hg (n=30) and increased with age; that of the 6-month-old rats was 196±10 mm Hg.
FIGURE 3. Photomicrograph of neuropeptide Y (NPY)-containing nerve fibers (whole-mount specimen) in distal region of mesenteric artery of a 7-week-old spontaneously hypertensive rat (SHR) (left panel) and that of a Wistar-Kyoto (WKY) rat (right panel) (peroxidase-antiperoxidase method). Density of NPY-containing nerve fibers of SHR is more dense than that of WKY rats (magnification ×300).

In the WKY rats, blood pressure at 7 weeks of age was 123±4 mm Hg (n=30), and 132±6 mm Hg was recorded at 6 months of age. Thus, the blood pressure of SHR was significantly higher than that of WKY rats (p<0.01).

The adrenergic nerve, which is vasoconstrictive, and the NPY-containing nerve exhibited dense innervation in the distal regions of mesenteric arteries of 7-week-old and 6-month-old SHR, as compared with findings in WKY rats of the same ages (Figures 2 and 3 and Table 1, p<0.01). In both SHR and WKY rat strains, the nerve densities at the middle and distal regions of both nerves were also higher than those at the proximal regions (Table 1). No difference was observed in the innervation densities of both nerves, regardless of the age and species. In both groups of SHR and WKY rats, the nerve densities of the adrenergic nerve and the NPY-containing nerve showed similar values in all the regions of the mesenteric arteries.

No difference was found in the innervation density of the cholinergic nerve, a vasodilative nerve, and the VIP- and SP-containing nerves between SHR and WKY rats at the respective ages (Figure 4 and Table 2). Among all age groups of SHR and WKY rats examined, there was no difference in density of any of the vasodilative nerves. The density of the cholinergic nerve was substantially sparse, whereas the density of the VIP-containing nerve was high (Figure 5). The SP-containing nerves had the same densities as the VIP-containing ones. Moreover, the densities of both nerves were higher in the middle and distal regions than in the proximal region.

Discussion

In studies on small arteries of the jejunum, Ichijima1 found that the SHR possessed higher densities of adrenergic nerve fibers even before the development of hypertension at 40–60 postnatal days, or at the early stage of hypertension 4–6 months after birth, in comparison with findings in the WKY rats. In observations on jejunal arteries, Scott and coworkers3,4 also pointed out that the adrenergic nerve fibers were more dense in SHR compared with WKY rats. Head et al2 reported that the noradrenaline content of the mesenteric arteries was greater in the SHR. It is thus considered histologically, as well as biochemically, that the dense distribution of adrenergic nerve in the peripheral arterial walls may be involved in the development and maintenance of hypertension in the SHR.

In addition to the aforementioned types of nerves, the presence of nerve fibers containing neuropep-

| Table 1. Densities of Vasoconstrictive Nerves in the Mesenteric Artery |
|-----------------|-----------------|-----------------|-----------------|
|                 | Proximal        | Middle          | Distal          |
|                 | 7W   | 6M   | 7W   | 6M   | 7W   | 6M   |
| Adn  SHR        | 52.3±3.4 | 53.4±2.8 | 117.6±2.4 | 116.6±2.8 | 123.1±4.8* | 122.8±3.4* |
| WKY  SHR        | 51.4±2.6 | 52.3±3.0 | 116.8±2.8 | 115.5±3.6 | 112.6±3.6* | 111.7±2.4* |
| NPY  SHR        | 53.2±2.5 | 52.4±2.6 | 115.2±3.4 | 113.7±2.4 | 127.3±5.4* | 126.5±3.4* |
| WKY  SHR        | 52.4±2.4 | 51.8±2.6 | 116.4±2.6 | 115.9±3.6 | 114.2±3.4* | 113.8±3.2* |

The number of vasoconstrictive nerve fibers crossing an imaginary 1 cm line drawn longitudinally along the mesenteric arteries. Value for each group represents the mean of measurements from six littersmates, with the range of standard deviation. Adn, adrenergic nerve fibers; NPY, neuropeptide Y-containing nerve fibers.

*p<0.01 between spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.
tides such as NPY, VIP, or SP has been clarified immunohistologically or by radioimmunoassay. Edvinsson et al. reported that although NPY constricted the cerebral arteries of cats, such constrictions exceeded the maximum constriction caused by noradrenaline and were equal in degree to those seen with serotonin. The constriction occurred in a dose-dependent and gradual manner and was prolonged. On the other hand, Lee et al. noted that VIP produced much the same dilative reaction even after removal of the endothelial cells and was not the endothelium-dependent dilative action seen with acetylcholine or SP.

Although it is assumed that nerves containing many newly discovered neuropeptides could be deeply involved in the autoregulation of local circulation, the manner in which the neuropeptide-containing nerves are actually involved in the development and maintenance of hypertension in SHR has remained unclear. Based on the results of our present study, we conclude 1) in the distal regions of the mesenteric arteries of 7-week-old and 6-month-old SHR, not only the adrenergic nerve, but also the NPY-containing nerve showed higher densities of innervation compared with findings in the WKY rat; 2) the cholinergic nerve, a vasodilative nerve, and the VIP- and SP-containing nerve showed no difference in innervation density between SHR and WKY rats. Thus, it was assumed that not only the adrenergic nerve but also the NPY-containing nerve could be closely linked to the development and maintenance of hypertension in the SHR.

In 1983 the immunoreactivity of NPY in the innervation nerves of cerebral and peripheral blood vessels was shown to be high. It was suggested that the distribution pattern of NPY might be much the same as the adrenergic nerve and that NPY might coexist with noradrenaline and adrenaline. When a superior cervical ganglionectomy was performed, the adrenergic nerve fibers distributed in cerebral blood vessels and the NPY-containing nerve fibers disappeared simultaneously; they were thus considered to be the same nerve fibers. There is, however, no documentation of the coexistence of both nerves in the mesenteric arteries. Although our study does not provide direct evidence for the coexistence of both these vasoconstrictive nerves in the mesenteric arteries, the distribution densities

**Figure 4.** Photomicrograph of substance P (SP)-containing nerve fibers (whole-mount specimen) in the distal region of mesenteric artery of 7-week-old spontaneously hypertensive rat (SHR) (left panel) and that of a Wistar-Kyoto (WKY) rat (right panel) (peroxidase-antiperoxidase method). Density of SP-containing nerve fibers of SHR is unchanged in comparison with that seen in the WKY rat (magnification x250).

**Figure 5.** Photomicrograph of cholinergic (left panel) and vasoactive intestinal polypeptide (VIP)-containing (right panel) nerve fibers (whole-mount specimen) in distal region of mesenteric artery of Wistar-Kyoto (WKY) rat at age 7 weeks. Kamovsky-Roots method (left) and peroxidase-antiperoxidase method (right). VIP-containing nerve fibers show relatively dense distribution in mesenteric artery whereas cholinergic nerve fibers are sparse. In addition, densities of both nerve fibers of spontaneously hypertensive rats are unchanged in comparison with those of WKY rats (magnification x250).
of both these nerves in the mesenteric arteries showed no difference in either SHR or WKY rats, and the distribution patterns of both nerves were similar. Thus, it is assumed that the two transmitters are possibly contained in an identical nerve fiber even under hypertensive conditions, and further that the increase of their synthesis may be involved in the development and maintenance of hypertension in SHR.

There is a report suggesting the coexistence of the cholinergic nerve and the VIP-containing nerve in cerebral arteries. However, not all cholinergic nerve fibers are positive for VIP immunoreactivity or vice versa. For example, after ligation of the cat mesenteric nerves a marked accumulation of VIP immunoreactivity was observed on the gut side, whereas very little acetylcholinesterase accumulated. Thus VIP-containing nerve fibers in the gut wall projecting to prevertebral ganglia seem to be noncholinergic nerve fibers. Based on the results of our present study, the density of distribution of the cholinergic nerve in the mesenteric arteries was found to be sparse, whereas that of the VIP-containing nerve was dense. We interpret our findings to mean that both vasodilative nerve fibers probably do not coexist in rat mesenteric arteries. However, since there is a rich innervation of both types of nerve fibers in mesenteric arteries of the bent-winged bats (K. Ando, unpublished data), these two nerves may have different systems in various species.

In the present study, a similar distribution and density of VIP- and SP-containing nerve fibers was noticed in the mesenteric arteries in both SHR and WKY rats. We found no direct evidence for the coexistence of VIP- and SP-containing nerve fibers; nevertheless our acquired evidence suggests that a possible coexistence would have to be ruled out.

Acknowledgment
We thank M. Ohara for helpful comments.

References

Table 2. Densities of Vasodilative Nerves in the Mesenteric Artery

<table>
<thead>
<tr>
<th>Group</th>
<th>Proximal</th>
<th>Middle</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7W</td>
<td>6M</td>
<td>7W</td>
</tr>
<tr>
<td>SHR</td>
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<tr>
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<td>42.0±2.8</td>
<td>57.4±3.4</td>
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<tr>
<td>SP</td>
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<tr>
<td>WKY</td>
<td>37.0±2.6</td>
<td>40.0±2.0</td>
<td>53.8±2.4</td>
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</tbody>
</table>

The number of vasodilative nerve fibers crossing an imaginary 1 cm line drawn longitudinally along the mesenteric arteries. Value for each group represents the mean of measurements from six littersmates, with the range of standard deviation.


**KEY WORDS** • mesenteric arteries • perivascular innervation • immunohistochemistry • spontaneously hypertensive rat
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