Characterization of Perivascular Nerve Distribution in Rat Mesenteric Small Arteries

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The distribution pattern of perivascular nerves in some branches of rat mesenteric arteries was studied. Mesenteric arteries isolated from 8-week-old Wistar rats were divided into the 1st-, 2nd-, and 3rd-order branches. The distribution of perivascular nerves in each branch was immunohistochemically evaluated using antibodies against neuropeptide Y (NPY), tyrosine hydroxylase (TH), calcitonin gene-related peptide (CGRP), substance P (SP), and neuronal nitric oxide synthase (nNOS). The density of NPY-, TH-, CGRP-, and SP-like immunoreactive (LI) nerves in the 2nd and 3rd branches was significantly greater than that in the 1st branch, and a negative relationship was found between nerve density and arterial diameter, except for TH-LI nerves. The density of NPY- and TH-LI nerves in all branches, which was similar, was greater than that of CGRP- (except for NPY-LI nerves in the 1st branch), SP-, or nNOS-LI nerves. Double immunostaining revealed that TH-LI nerves made contact with nNOS-LI, CGRP-LI, and SP-LI nerves and that CGRP-LI nerves made contact with TH-, NPY-, or nNOS-LI nerves, while TH-LI and CGRP-LI nerves nearly merged with NPY-LI and SP-LI nerves, respectively. These results suggest that the each branch of mesenteric arteries is densely innervated by vasoconstrictor nerves containing NPY, TH, and vasodilator CGRP nerves. They also suggest that the intense density of perivascular nerves in the 2nd and 3rd branches may contribute to maintaining vascular tone.

Key words adrenergic nerve; nonadrenergic noncholinergic nerve; nerve distribution; rat mesenteric small artery

Rat mesenteric arteries are innervated by perivascular nerves that play an important role in the maintenance of vascular tone and regulation of organ and tissue blood flow. With few exceptions, postganglionic adrenergic nerves constitute the most significant effenter neural pathway to blood vessels. The degree of microcirculation control exerted by adrenergic nerves depends, in part, on the distribution pattern of these nerves to the various segments comprising the microvascular network. The functional significance of microcirculation control via adrenergic nerves may vary among different organs and between different segments of the microvascular network in any given tissue. Resistance vessels, such as the mesenteric arteries, are known to be innervated by many perivascular nerves, such as sympathetic adrenergic nerves, and non-adrenergic non-cholinergic (NANC) nerves including CGRPergic nerves and nitric oxide (NO) nerves. Our previous studies also revealed the elimination of CGRPergic nerve function augmented adrenergic nerve-mediated vasoconstriction, and, conversely, adrenergic nerves via presynaptic norepinephrine release inhibited the neurogenic release of calcitonin gene-related peptide (CGRP) from the nerve, thereby decreasing CGRPergic nerve function. We also previously demonstrated adrenergic and NO nerves innervating rat mesenteric arteries were involved in the modulation of adrenergic neurotransmission. These findings of neuronal interactions led to the hypothesis that perivascular adrenergic and NANC nerves innervating mesenteric arteries make axo-axonal interactions. Although the vascular tone of rat mesenteric small artery categorized as the muscular resistance artery depends mainly on the perivascular nerve activity, it is not known whether the distribution of perivascular nerves depends on vascular size, resulting in the large change of vascular tone. Furthermore, the different populations of perivascular nerves in various diameter of small artery have not been fully investigated. Since very few systematic studies have described perivascular innervation including adrenergic nerves of the small circulation in any organs, the present study was designed to characterize populations of perivascular nerves in the different size of the mesenteric small artery and the innervation pattern between perivascular nerves.

MATERIALS AND METHODS

Animals Eight-week-old Wistar rats (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were used (n=30) in this study. Animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C with 50±10% relative humidity and a 12-h light/12-h dark cycle (lights on at 8:00 a.m.). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 115) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

Immunohistochemical Study Animals were anesthetized...
with 50 mg/kg pentobarbital-Na (Dainippon-Sumitomo Pharmaceutical Co., Tokyo, Japan). The superior mesenteric artery was cannulated with polyethylene tubing and phosphate-buffered saline (PBS) was infused to remove blood followed by injection of Zamboni solution. The mesenteric vascular bed was then removed together with the intestines. The 1st (300–400 µm in diameter), 2nd (250–300 µm), and 3rd branches (200–250 µm) of the mesenteric artery were removed and immersion-fixed in Zamboni solution for 48 h. After fixation, each artery was repeatedly rinsed in PBS, immersed in PBS containing 0.5% TritonX-100 overnight, and incubated with PBS containing normal goat serum (1:100) for 60 min. The tissue was then incubated with rabbit polyclonal anti-tyrosine hydroxylase (TH) serum (1:500) (Chemicon International, Inc., Temecula, CA, U.S.A.), rabbit anti-neuropeptide Y (NPY) serum (1:300) (Phoenix Pharmaceuticals Inc., Belmont, CA, U.S.A.), rabbit polyclonal anti-CGRP serum (Biomol International, Inc., Kelayres, PA, U.S.A.) (1:500), anti-substance P (SP) serum (1:200) (Biomol International, Inc.), or anti-neuronal nitric oxide synthase (nNOS) serum (1:500) (Zymed Laboratories, South San Francisco, CA, U.S.A.) for 72 h at 4°C. The sites of the antigen-antibody reaction were revealed and the artery was observed as previous study.

Double Immunostainings Mesenteric arteries were incubated with PBS containing 1% normal goat serum, 1% bovine serum albumin, and 0.03% Triton X-100 for 1 h. These sections were then incubated with rabbit anti-TH serum (1:500) (Chemicon International, Inc.), rabbit anti-NPY serum (1:300) (Phoenix Pharmaceuticals Inc.), rabbit anti-nNOS serum (1:500) (Zymed Laboratories), rabbit anti-SP serum (1:200) (Biomol International, Inc.), or rabbit anti-CGRP serum (1:300) (Biomol International, Inc.) at 4°C for 2 d, followed by an incubation with guinea-pig anti-CGRP serum (1:300) (American Research Products, Inc., Waltham, MA, U.S.A.) or mouse anti-TH serum (1:100) (Chemicon International, Inc.) at 4°C for 2 d. Following washes in PBS, the sections were incubated for 2 h with rhodamine-conjugated goat F(ab')2 fraction anti-guinea pig immunoglobulin G (IgG) (1:100) (ICN Pharmaceuticals, Inc.) at 4°C, and then incubated with fluorescein-5-isothiocyanate (FITC)-conjugated rabbit IgG fractions (1:100) (ICN Pharmaceuticals, Inc.) at 4°C for 1 h. The samples were then observed under a confocal laser scanning microscope (CLSM 510, Carl Zeiss) with an exciting filter system (458/488 nm for FITC) and emission filter system (543 nm for rhodamine).

Immunohistochemical Analysis The immunostaining density of all nerves, which were isolated, fixed, and immunostained at the same time on the same day, was analyzed using the same method. The 1st branch of mesenteric arteries was used as a control for the intensity in each experiment. Confocal projection images of immunostaining, which were patched together with 8–10 overlapping images (0.1 µm scanning each), were magnified at 20×, digitized as TIF images using a digital camera system (Olympus SP-1000, Olympus, Tokyo, Japan) for the quantitative evaluation. The measured field of 100 µm x 100 µm (10000 µm², which contained the adventitia layer including immunostained perivascular nerves) was randomly selected on magnified images of the whole mount.

Fig. 1. Typical Images Showing Immunopositive Nerves of NPY (A), TH (B), nNOS (C), CGRP (D), and SP (E) in the First (1st), Second (2nd), and Third (3rd) Branches of Rat Mesenteric Arteries

The horizontal bar in each image indicates 100 µm.
For each animal, the average density in 3 arteries from each part of the vascular bed was calculated and also determined the number of all nerves.4)

Statistical Analysis All data were expressed as the mean ± standard error of the mean (S.E.M.). An ANOVA followed by Tukey’s test or Dunnett’s test was used to determine significance where appropriate. A correlation analysis was carried out using Spearman’s correlation test. A value of p<0.05 was considered significant.

RESULTS

Distribution of Perivascular Nerves in 1st, 2nd, and 3rd Branches of Mesenteric Arteries NPY-LI, TH-LI, nNOS-LI, CGRP-LI, and SP-LI nerves formed a plexus around outside of all branches of mesenteric arteries (Fig. 1). The densities of NPY-LI, TH-LI, nNOS-LI (only in 3rd branch), SP-LI, and CGRP-LI nerves in the 2nd and 3rd branches were markedly greater than those in the 1st branch (Fig. 2). Significant positive correlations were observed between the densities and numbers of all nerves in the 1st branch (NPY-LI, \( p<0.01, R^2=0.9558 \); TH-LI, \( p<0.05, R^2=0.9300 \); nNOS-LI, \( p<0.05, R^2=0.9736 \); CGRP-LI, \( p<0.05, R^2=0.9710 \); SP-LI, \( p<0.05, R^2=0.8926 \)) (Fig. 3A), 2nd branches (NPY-LI, \( p<0.01, R^2=0.9602 \); TH-LI, \( p<0.05, R^2=0.9638 \); nNOS-LI, \( p<0.05, R^2=0.9480 \); CGRP-LI, \( p=0.0639, R^2=0.7298 \); SP-LI, \( p=0.0639, R^2=0.8556 \)) (Fig. 3B), and 3rd branches (NPY-LI, \( p<0.01, R^2=0.9393 \); TH-LI, \( p<0.05, R^2=0.9517 \); nNOS-LI, \( p<0.05, R^2=0.9907 \); CGRP-LI, \( p=0.0639, R^2=0.7701 \); SP-LI, \( p<0.05, R^2=0.9321 \)) (Fig. 3C).

A significant negative relationship was also observed between the density and arterial diameter of NPY-LI, nNOS-LI, CGRP-LI, and SP-LI nerves in all branches, but not TH-LI nerve (Fig. 4). Figure 5 shows a comparison of the distribution density of all nerves in the 1st, 2nd, and 3rd branches of mesenteric arteries. The densities of NPY-LI and CGRP-LI nerves were similar to those of TH-LI and SP-LI nerves in all branches, respectively. In contrast, the densities of nNOS-LI, CGRP-LI, and SP-LI nerves in the 2nd and 3rd branches were significantly smaller than those of NPY-LI and TH-LI nerves. However, the density of CGRP-LI nerve in the 1st branches was no different from that of NPY-LI nerve.
Fig. 3. Relationship between Number and Density of Various Immunopositive Nerves in 1st (A), 2nd (B), and 3rd (C) Branches of Rat Mesenteric Arteries

Data of each nerve were statistically analyzed by Spearman's test.
LI nerve exhibited the smallest distribution density among immunostained perivascular nerves in all branches.

**Characterization of Perivascular Nerves in 1st, 2nd, and 3rd Branches of Mesenteric Arteries**  Double immunohistochemistry revealed TH-LI nerves almost merged with NPY-LI nerves (Figs. 6A, 7D). However, TH-LI nerves came into close contact, but did not merge, with most nNOS-LI (Fig. 6B), CGRP-LI (Fig. 6C), and SP-LI (Figs. 6D, 7B) nerves in the 2nd and 3rd branches.

Most CGRP-LI nerves made close contact, but did not merge with TH-LI (Fig. 8A), NPY-LI (Fig. 8B), and most nNOS-LI (Figs. 7A, 8C) nerves. However, CGRP-LI nerves almost merged with SP-LI nerves (Figs. 7C, 8D).

**DISCUSSION**

We previously reported that rat mesenteric arteries were densely innervated by sympathetic vasoconstrictor NPY-LI and NANC vasodilator CGRP-LI nerves, which contributed to vascular tone regulation. The present immunohistochemical study demonstrated the innervation pattern of perivascular nerves in which, in addition to NPY-LI and CGRP-LI nerves, TH-LI, nNOS-LI, and SP-LI nerves had a different population along rat mesenteric arteries. TH is a rate-limiting enzyme for noradrenaline synthase and contained in sympathetic nerves. NPY, which is co-localized with noradrenaline, is also a sympathetic neuropeptide that induces vasoconstriction, the hypertrophic and mitogenic effects observed in vascular smooth muscle and endothelial cells. As shown in Fig. 7, TH-LI and NPY-LI nerves overlapped in the sympathetic nerves innervating the mesenteric arteries. This result was supported by previous findings in which TH-LI nerves co-existed with NPY-LI nerves in the skeletal muscle arteries of various kinds of animals, such as rabbits, dogs, cats, and so on.
Accumulating evidence shows that CGRP-LI, nNOS-LI, and SP-LI nerves are NANC nerves that provoke vasodilation mediated by released neurotransmitters, such as CGRP, NO, and SP, respectively, and also regulate tissue blood flow by reciprocally interacting with perivascular sympathetic nerves.8) However, the innervation pattern of these NANC perivascular nerves currently remains unknown in small arteries.

The present immunohistochemical results obtained by investigating the distribution pattern of perivascular nerves including vasoconstrictor nerves (NPY-LI and TH-LI nerves) and vasodilator nerves (CGRP-LI, nNOS-LI, and SP-LI nerves) in the 1st, 2nd, and 3rd branches of rat mesenteric arteries revealed a significant difference associated with arterial diameter. All perivascular nerves detected, except for TH-LI nerves, were distributed in an inversely-related manner with arteriolar diameter, indicating that 3rd branches were densely innervated perivascular nerves than the other branches. Therefore, it is highly possible that perivascular nerves are closely involved in the tone regulation of arteries with smaller diameter, especially resistance arteries including 2nd and 3rd branches, and control of blood flow more than regulatory mechanisms by endothelial cells. These results were supported by previous findings in which cholinergic and noradrenergic nerves innervate small-diameter vessels of rat coronary with a negative correlation being observed between the innervation density and vessel diameter.10)

In all branches without the 1st branch, the density of NPY-LI and TH-LI nerves was markedly higher than that of all vasodilator nerves. Interestingly, the distribution of nNOS-LI nerves was the smallest among the other perivascular nerves. The present results clearly demonstrated the first evidence that the density of vasoconstrictor and vasodilator nerves in all branches were almost the same, suggesting no change in the importance of the different nerve populations in regulating vascular tone.

Kawamura et al. reported that spontaneously hypertensive rats exhibited the denser innervation of adrenergic nerves, but not vasoactive intestinal polypeptide (VIP)- or SP-containing nerves, in the distal regions of mesenteric arteries than that in control normotensive rats at the same ages.11) Our previous studies also revealed that the abnormal innervation of perivascular nerves in mesenteric arteries, which was induced by exposure to chronic hyperinsulinemia, altered the neuronal regulation of vascular tone, leading to hypertension in type 2 diabetes model rats.12,13) These findings and the present results suggest that an altered pattern of perivascular nerve innervation could affect the development and maintenance of cardiovascular diseases.

The present double immunostaining study demonstrated that adrenergic TH-LI nerves closely contacted vasodilator nerves including nNOS-LI, CGRP-LI, and SP-LI nerves. This result strongly suggests that vasoconstrictor and vasodilator nerves make an axo-axonal interaction to regulate vascular tone. Furthermore, most CGRP-LI nerves had close contacts with most nNOS-LI nerves, suggesting that vasodilator nerves also interacted with each other. CGRP and SP, as a neuropeptide, have been shown to co-localize in the same sensory neuron. This is supported by the present double immunostaining result in which CGRP-LI almost merged with SP-LI nerves. However, previous studies reported that CGRP-LI and SP-LI nerves were distributed differently and played a neuro-modulatory role in nociception and peripheral cardiovascular responses in several species.14,15) The function of SP-LI nerves in mesenteric arteries has not yet been fully elucidated, however, a very close innervation pattern of SP-LI and CGRP-LI nerves was observed in rat mesenteric arteries.

On the other hand, Schwarz et al. demonstrated the co-existence of nNOS with TH immunoreactivity in numerous nerves distributed in the rat atria,16) whereas Jew et al. detected the...
Fig. 6. Double Immunostaining Images Showing TH-LI- (Left; Red) and NPY (A)-LI-, nNOS (B)-LI-, CGRP (C)-LI-, or SP (D)-LI Nerves (Middle; Green) in the 2nd and 3rd Branch Arteries
Right images indicate a superimposition of the left and middle images. The horizontal bar in each image indicates 50 µm.

Fig. 7. Magnified Double Immunostaining Images Showing CGRP-LI- and nNOS-LI- (A) or SP-LI- (C), TH-LI- and SP-LI- (B) or NPY-LI- (D) Nerves in Rat Mesenteric Arteries
The horizontal bar in each image indicates 20 µm.

nNOS-LI and TH-LI nerve innervations in separate areas of the rat heart mitral valve. Thus, limited immunohistochemical evidence is available for nNOS in cardiac sympathetic nerve terminals. However, Hatanaka et al. showed that NO released from perivascular capsaicin-sensitive nerves, presynaptically and indirectly, decreased neurogenic norepinephrine release in order to modulate adrenergic neurotransmission. This finding and the present results that TH-LI nerves were in close contact with nNOS-LI nerves indicate that nNOS-LI nerves play a critical role in sympathetic nerve function in rat mesenteric arteries.

The studies using human mesenteric vessels showed abnormalities in the perivascular innervation of patients with the inflammatory bowel disease (IBD) in which both density of sympathetic nerves in arteries and veins and CGRP nerve in veins were markedly increased, suggesting an important role in the pathogenesis and/or development of IBD. In obese humans, sympathetic outflows to the kidney and skeletal muscle have been reported to be augmented, and there exists a close correlation between sympathetic hyperactivity and structural changes, resulting in arterial stiffness, endothelial dysfunction, cardiac hypertrophy and renal damage. The sympathetic nerve-mediated vasoconstriction and the density of perivascular sympathetic nerves in the rat mesenteric artery were enhanced by diet-induced obesity. Although the increase in the activity or innervation of sympathetic nerves is likely to contribute to hypertension, the species difference in the function or distribution of perivascular nerves between the
human and rat is not fully determined yet.

These results suggest that the rat mesenteric small artery has almost same innervation patterns between vasoconstrictor nerves containing CGRP, nNOS, and SP in all branches. Furthermore, an augmented distribution of perivascular nerves in the area of arteries with smaller diameter is likely to be associated with a physiological and/or pathological significance of vascular tone regulatory mechanism via perivascular nerves.

Conflict of Interest The authors declare no conflict of interest.

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