Neurogenic Vascular Responses in Male Mouse Mesenteric Vascular Beds

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Abstract. Rat mesenteric arteries were maintained by both adrenergic vasoconstrictor nerves and calcitonin gene–related peptide (CGRP) vasodilator nerves. However, functions of these nerves in a pathophysiological state have not fully been analyzed. The use of disease models developed genetically in mice is expected to clarify neural function of perivascular nerves. Thus, we investigated basic mouse vascular responses. Mesenteric vascular beds isolated from male C57BL/6 mouse were perfused with Krebs solution and perfusion pressure was measured. Periarterial nerve stimulation (PNS, 8 – 24 Hz) induced frequency-dependent vasoconstriction, which increased flow rate–dependently. PNS-induced vasoconstriction was abolished by tetrodotoxin (neurotoxin) and guanethidine (adrenergic neuron blocker) and blunted by prazosin (α1-adrenoceptor antagonist). Injection of norepinephrine caused vasoconstriction, which was abolished by prazosin. In preparations with active tone, PNS (1 – 8 Hz) induced frequency-dependent vasodilation, which was inhibited by tetrodotoxin, capsaicin (CGRP depletor), and CGRP8-37 (CGRP-receptor antagonist). Injections of CGRP, acetylcholine, and sodium nitroprusside induced vasodilatations. Vasodilator response to CGRP was inhibited by CGRP8-37. Immunohistochemical study showed innervation of tyrosine hydroxylase- and CGRP-immunopositive fibers in mesenteric arteries and veins. These results suggest that male mouse mesenteric vascular beds are useful for studying neural regulation of mesenteric arteries, which are innervated by adrenergic and CGRPergic nerves regulating vascular tone.

Keywords: adrenergic vasoconstriction, calcitonin gene–related peptide (CGRP), CGRPergic vasodilation, mesenteric vascular bed, perivascular innervation

Introduction

It is widely accepted that vascular adrenergic nerves regulate the tone of the peripheral vascular system and maintain blood pressure. When vascular adrenergic nerves are stimulated, peripheral vascular tone is increased mainly by released norepinephrine and partially by co-released neuropeptide Y and ATP (1, 2). Adrenergic nerves have been shown to be densely distributed in rat mesenteric arteries by immunohistochemical staining (1, 3), reaction to periarterial nerve stimulation (PNS) (4, 5), and measurements of the amount of norepinephrine released (6). However, evidence has accumulated that blood vessels are innervated by non-adrenergic non-cholinergic (NANC) nerves (4, 7). We reported that PNS in rat mesenteric vascular beds produced NANC nerve-mediated vasodilation (4, 8), which is mediated by calcitonin gene–related peptide (CGRP), a potent vasodilator neurotransmitter (4). Moreover, immunohistochemical study demonstrated that CGRP-containing (CGRPergic)
nerves were distributed in rat mesenteric arteries (1, 4). Previous reports showed that NANC CGRPergic nerves are involved in the regulation of vascular tone along with adrenergic nerves (9). Additionally, impaired functioning of perivascular NANC and adrenergic nerves has been demonstrated using various pathological models including insulin-resistant rats (10), spontaneously hypertensive rats (8) and renal hypertensive rats (11), obese rats (12), and a model of perivascular nerve degeneration (1). Various pathophysiological models have also been established in mice through genetic engineering and so the functions of perivascular nerves in a pathological state are often analyzed using gene-knockout and gene–over-expressing mice. However, very little has been reported on the neuronal regulation of mouse mesenteric vascular beds. Thus, the present study investigated mouse preparations of mesenteric vascular beds and determined basic conditions for perfusion and electrical field stimulation. Additionally, we studied whether neurogenic vascular responses via adrenergic nerves and CGRPergic nerves are observed in male mouse vascular beds as in rat preparations.

Materials and Methods

Animals

Male C57BL/6 mice weighing 25 – 30 g (8 – 9-week-old) (purchased from Shimizu Laboratory Supplies Co., Ltd., Shizuoka) were used in the present study. All animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University under a controlled ambient temperature of 22°C ± 2°C with 50% ± 10% relative humidity and a 12-h light / 12-h dark cycle (light on 08:00 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 Safe-keeping of Animals No. 6. Every effort was made to minimize the number of animals used and their suffering.

Perfusion of mesenteric vascular beds and measurement of perfusion pressure

The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the mesenteric vascular beds were isolated and prepared for perfusion according to the previously described method (4, 9, 13). Briefly, a piece of polyethylene tubing (0.78 mm in outer diameter), stretched thin by heating, was inserted into the superior mesenteric artery through the abdominal aorta, and Krebs-Ringer bicarbonate solution (Krebs solution) (see below) was gently flushed to eliminate blood from the vascular bed. After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The isolated mesenteric vascular bed was then placed in a water-jacketed organ bath maintained at 37°C and perfused with Krebs solution at a constant flow rate of 1 to 5 mL/min with a peristaltic pump (model AC-2120; Atto Bioscience, Rockville, MD, USA) and superfused with the same solution at a rate of 0.5 mL/min to prevent drying. The Krebs solution was bubbled with a mixture of 95% O2 and 5% CO2 before passing through a warming coil at 37°C. The Krebs solution had the following composition: 119.0 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl2, 1.2 mM MgSO4, 25.0 mM NaHCO3, 1.2 mM KH2PO4, 0.03 mM EDTA-2Na, and 11.1 mM glucose, pH 7.4. Changes in perfusion pressure were measured with a pressure transducer (model TP-200T; Nihon Kohden, Tokyo) and recorded using a pen recorder (model U-228; Nippon Kayaku, Tokyo).

PNS and injections of norepinephrine, acetylcholine (ACh), sodium nitroprusside (SNP), and CGRP

Electrical field stimulation of perivascular nerves at 1 – 24 Hz was applied using bipolar platinum ring-shaped electrodes between which was placed the superior mesenteric artery. Rectangular pulses of 10 – 50 μs and supramaximal voltage (50 V) were applied for 30 s using an electronic stimulator (model SEN 3301, Nihon Kohden). The interval between stimuli was 5 to 10 min. The next stimulant was delivered when the perfusion pressure returned to pre-stimulation levels.

Norepinephrine, ACh, SNP, and CGRP were directly injected into the perfusate proximal to the arterial cannula with an infusion pump (model B-25326; Harvard Apparatus, Inc., Holliston, MA, USA). A volume of 60 μL was injected during a period of 12 s.

Experimental protocols for determination of flow rate with resting tone

The preparation was perfused for 30 min to stabilize perfusion pressure with resting tone. Changes in perfusion pressure were measured every 30 min when the flow rate was changed to 1, 2, 3, 4, and 5 mL/min.

To assess vascular tone, Krebs solution containing the vasodilator papaverine at 100 μM was perfused at a flow rate of 1 to 5 mL/min and changes in perfusion pressure were measured.

To assess vascular responsiveness, responses to PNS (voltage: 50 V; pulse duration: 30 μs; frequency: 8, 12,
16, 20, and 24 Hz) were measured at each flow rate without papaverine perfusion.

**Experimental protocols for determination of stimulus conditions with resting tone**

Vascular responses induced by PNS were observed by changing pulse duration from 10 to 50 \( \mu \text{s} \) at a 3 mL/min flow rate. To identify neurogenic responses, these responses were examined in the presence of the neurotoxin tetrodotoxin (Na\(^+\)-channel blocker, 0.5 \( \mu \text{M} \)) or the adrenergic neuron blocker guanethidine (5 \( \mu \text{M} \)). Moreover, the involvement of \( \alpha_1 \)-adrenoceptors in the responses to PNS and bolus injection of norepinephrine was examined in the presence of the \( \alpha_1 \)-adrenoceptor antagonist prazosin (5 \( \mu \text{M} \)). Tetrodotoxin, guanethidine, or prazosin was perfused from 10 min before PNS or norepinephrine injection was tested to the end of the experiment.

**Experimental protocols for PNS and injections of vasodilator agents with active tone**

The preparations had no active resting tone unless adrenergic nerves were stimulated by PNS or adrenoceptors were activated by an adrenoceptor agonist. Therefore, to observe the vasodilator responses, the preparation was continuously perfused with methoxamine (7 \( \mu \text{M} \)) to produce and maintain active resting tone, and guanethidine (5 \( \mu \text{M} \)) was added to block adrenergic neurotransmission. After the perfusion pressure was stabilized, PNS and injections of ACh, SNP, and CGRP were performed in the presence of Krebs solution containing methoxamine (7 \( \mu \text{M} \)) and guanethidine (5 \( \mu \text{M} \)) or tetrodotoxin (0.5 \( \mu \text{M} \)), which was perfused from 10 min before PNS or norepinephrine injection was tested to the end of the experiment.

In another series of experiments, the involvement of CGRP\(_1\) receptors in PNS-induced vasodilation was examined using the CGRP\(_1\)-receptor antagonist CGRP8-37. After vascular responses to PNS and the first injection of CGRP were obtained as controls, Krebs solution was switched to Krebs solution containing methoxamine (7 \( \mu \text{M} \)), guanethidine (5 \( \mu \text{M} \)), and CGRP8-37 (1 \( \mu \text{M} \)), and 10 min after perfusion, a second injection of CGRP and PNS was carried out.

At the end of each experiment, the preparation was perfused with papaverine (100 \( \mu \text{M} \)) to cause complete relaxation. Vasodilator activity was expressed as a percentage of the perfusion pressure at maximum relaxation induced by papaverine.

**In vitro capsaicin treatment**

In vitro capsaicin treatment was performed to deplete perivascular CGRPergic nerves according to a method described previously (4, 14). The preparation was perfused with Krebs solution containing capsaicin (10 \( \mu \text{M} \)) for 30 min and then rinsed with capsaicin-free Krebs solution for 30 min. Thereafter, active tone was produced by perfusing Krebs solution containing methoxamine and guanethidine, and PNS and injections of ACh, CGRP, and SNP were carried out.

**Immunohistochemistry**

The animals were anesthetized with a large dose of sodium pentobarbital (50 mg/kg, intraperitoneally). The superior mesenteric artery was cannulated with polyethylene tubing and Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) was infused. Then, the mesenteric vascular bed was removed together with the intestine as described previously (1, 15). The second or third order branch of the mesenteric artery and vein proximal to the intestine was removed and immersion-fixed in the Zamboni solution for 48 h. After fixation, the artery and vein were repeatedly rinsed in phosphate-buffered saline (PBS), immersed in PBS containing 0.5% TritonX-100 overnight, and incubated with PBS containing normal goat serum (1:100) for 60 min. The artery and vein were then incubated with guinea-pig polyclonal anti-CGRP antiserum (American Research Products, Inc., Belmont, MA, USA) at a dilution of 1:100 or rabbit polyclonal anti-tyrosine hydroxylase (TH) IgG (Chemicon international, Inc., Temecula, CA, USA) at a dilution of 1:500 for 72 h at 4°C. After the incubation, the artery and vein were washed in PBS and the sites of antigen-antibody reaction were detected by incubation with Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:200) or Alexa Fluor 555–conjugated goat anti-rabbit IgG (1:1000) (Invitrogen, Carlsbad, CA, USA) for 60 min. Thereafter, the artery and vein were thoroughly washed in PBS, mounted on a slide, and cover-slipped with glycerol/PBS (2:1 v/v). Immunofluorescence was detected and processed using a confocal laser microscope (CLSM510; Carl Zeiss, Tokyo) fitted with a \( \times \) 20 objective under the 488 nm spectrum of an argon laser.

In another series of experiments, in vitro adrenergic denervation was carried out by incubation with 6-hydroxydopamine (6-OHDA) as described by Zhang et al. (16). The isolated mesenteric vascular bed was incubated in Krebs solution containing 6-OHDA (4 mM) and 40 \( \mu \text{M} \) glutathione for 30 min, twice with a 30 min-interval in 6-OHDA-free Krebs solution. Thereafter, the small arteries were removed, immersion-fixed with Zamboni solution, and subjected to immunohistochemistry. To avoid any misinterpretation of immunopositive reactions or artifacts, immunostaining with or without the primary
or secondary antibody was first examined as a control. However, no immunopositive reaction was observed when the primary or secondary antibody was omitted.

In another series of experiments, in vitro depletion of CGRPergic nerves was performed according to the method described previously (4, 8). The isolated mesenteric vascular bed was incubated with Krebs solution containing capsaicin (40 μM) for 30 min and then rinsed with capsaicin-free Krebs solution. The small arteries were removed, immersion-fixed with Zamboni solution, and subjected to immunohistochemistry.

At least 3 preparations were examined for immunostaining in each group.

**Statistical analyses**

The experimental results are presented as the mean ± S.E.M. Statistical analysis was performed by Student’s paired or unpaired t-test and a two-way analysis of variance and the Bonferroni post-tests. A P value less than 0.05 was considered statistically significant.

**Drugs**

The following drugs and agents were used: acetylcholine chloride, norepinephrine hydrochloride and tetrodotoxin (Daiichi Sankyo Co., Tokyo); capsaicin, glutathione, guanethidine sulphate, 6-OHDA hydrobromide, prazosin, and SNP (Sigma-Aldrich Japan, Tokyo); pentobarbital sodium salt (Tokyo Chemical Industry Co., Tokyo); CGRP and CGRP8-37 (Peptide Institute, Osaka); methoxamine hydrochloride (Nihon Shinyaku Co., Kyoto); and papaverine hydrochloride (Dainippon-Sumitomo Co., Osaka). All drugs, except for capsaicin, were dissolved in pure water and diluted with Krebs solution. Capsaicin was dissolved in 50% ethanol and diluted with Krebs solution (final ethanol concentration, 0.4 mg/mL). Acetylcholine, CGRP, and SNP were diluted with Krebs solution containing 7 μM methoxamine and 5 μM guanethidine when injected directly.

**Results**

**Determination of optimal perfusion flow rate in mouse mesenteric vascular beds**

As shown in Fig. 1, A and B, the increase in rate of perfusion from 1 to 5 mL/min raised the mean basal perfusion pressure in a flow rate–dependent manner. A flow rate of 4 and 5 mL/min caused spontaneous fluctuations in basal perfusion pressure without any stimulation. As shown in Fig. 1, A and C, PNS (voltage, 50 V; pulse duration, 30 μs; frequency, 8 to 24 Hz) of the mesenteric vascular beds with resting tone produced a frequency-dependent increase in perfusion pressure due to vasoconstriction. The PNS-induced vasoconstriction was augmented by increasing the flow rate (Fig. 1C). However, a flow rate of more than 3 mL/min did not alter the PNS-induced response. From these findings, 3 mL/min was taken as the optimal rate to observe vascular responses and used in subsequent experiments.

**Fig. 1.** Determination of optimal flow rate based on PNS-induced responses in male mouse perfused mesenteric vascular beds. A typical recording (A) showing PNS-induced vasoconstriction of a flow rate of 1 – 5 mL/min. A bar graph (B) and a line graph (C) show flow rate–induced changes in resting perfusion pressure and PNS-induced vasoconstrictor response. Each point indicates the mean ± S.E.M. *P < 0.05 compared with 1 mL/min group using a two-way ANOVA and Bonferroni’s multiple test.
As shown in Table 1, the vasodilator papaverine did not affect resting perfusion pressure raised by increasing the flow rate from 1 to 5 mL/min.

**Influence of pulse duration and effect of tetrodotoxin on frequency-dependent vasoconstrictor responses to PNS**

As shown in Fig. 2, A – C, PNS with 10-μs pulses (voltage, 50 V; frequency, 8 to 24 Hz) in preparations with resting tone caused little vasoconstriction, while pulses of 30 and 50 μs induced a frequency and pulse duration–dependent vasoconstriction (Fig. 2: B and C).

As shown in Fig. 2, D and E, the neurotoxin tetrodotoxin (0.5 μM) abolished the vasoconstrictor response to PNS with 30-μs pulses. However, the toxin inhibited, but did not abrogate, the response to 50-μs pulses and small vasoconstrictions were observed, indicating that pulses lasting more than 50 μs induced tetrodotoxin-insensitive vasoconstriction. As shown in Fig. 2F, tetrodotoxin eliminated the vasoconstricter responses to 30-μs pulses at all frequencies. From these findings, 30 μs was taken as the optimal duration for pulses to evoke neurogenic vascular responses and used for subsequent experiments.

**Effect of guanethidine on vasoconstrictor responses to PNS**

We examined the involvement of perivascular adrenergic nerves in PNS-induced vasoconstriction using guanethidine, a sympathetic adrenergic neuron blocker. As shown in Fig. 3, guanethidine (5 μM) abolished the vasoconstrictor response to PNS (50 V, 30-μs pulse duration, 8 to 24 Hz). However, the vascular response at 50 μs was not abolished and some vasoconstriction was observed even in the presence of 10 μM guanethidine (data not shown).

**Effect of prazosin on vasoconstrictor responses to PNS and injections of norepinephrine**

In a preparation with resting tension, as shown in Fig. 4A, PNS-induced vasoconstriction was markedly re-

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**Table 1.** Effect of papaverine (PPV, 100 μM) on flow rate (1 to 5 mL/min)–induced changes in resting perfusion pressure of male mouse mesenteric vascular beds

<table>
<thead>
<tr>
<th>Flow rate of perfusion (mL/min)</th>
<th>n</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>18.8 ± 1.9</td>
<td>31.5 ± 2.4</td>
<td>42.0 ± 2.3</td>
<td>52.0 ± 2.8</td>
<td>60.8 ± 3.3</td>
</tr>
<tr>
<td>Papaverine (100 μM)</td>
<td>4</td>
<td>18.0 ± 1.9</td>
<td>30.8 ± 2.8</td>
<td>40.8 ± 2.6</td>
<td>50.5 ± 2.7</td>
<td>59.5 ± 3.0</td>
</tr>
</tbody>
</table>

PPV was continuously perfused with during the experiment. Data are reported as the mean ± S.E.M.

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**Fig. 2.** Typical recordings showing influence of pulse duration and tetrodotoxin on frequency-dependent vascular responses induced by periarterial nerve stimulation (PNS: 8, 12, 16, 20, and 24 Hz) in male mouse perfused mesenteric vascular beds with resting tension. Panels A, B, and C indicate the responses to 10-, 30-, and 50-μs pulse duration, respectively. Panels D and E show responses to 30- and 50-μs pulse duration in the presence of tetrodotoxin (0.5 μM), respectively. A bar graph (F) shows the effect of tetrodotoxin on the response to 30-μs pulse duration. Each bar indicates the mean ± S.E.M. *P < 0.05, **P < 0.01, compared with no-treatment.
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Fig. 3. Typical recordings (A, B) and a bar graph (C) showing the effect of guanethidine (5 μM) on vascular responses induced by periarterial nerve stimulation (PNS) in male mouse perfused mesenteric vascular beds with resting tension. The PNS at 8, 12, 16, 20, and 24 Hz and 30-μs pulse duration was given for 30 s. Each bar indicates the mean ± S.E.M. *P < 0.05, **P < 0.01, compared with no treatment.

Fig. 4. A typical recording (A) and bar graphs (B and C) showing the effect of prazosin (5 μM) on vascular responses induced by periarterial nerve stimulation (PNS; 12, 16, and 20 Hz) (B) and injections of norepinephrine (NE, 5 and 10 nmol) (C) in male mouse perfused mesenteric vascular beds with resting tension. Each bar indicates the mean ± S.E.M. **P < 0.01, compared with no treatment.

duced, but not completely abolished, by prazosin (5 μM) (Fig. 4B).

As shown in Fig. 4A, injections of norepinephrine caused an increase in perfusion pressure due to vasoconstriction in a dose-dependent manner. Also, injections of Krebs solution without norepinephrine slightly increased perfusion pressure, indicating a change in pressure due to the direct injection of the solution into the vascular bed. As shown in Fig. 4, A and C, prazosin abolished the norepinephrine-induced vasoconstriction, but not Krebs solution–induced pressure increase, which was similar to that in the absence of prazosin.
Vasodilator responses to PNS and injections of ACh, SNP, and CGRP and effect of tetrodotoxin in mesenteric vascular beds with active tone

To observe vasodilation, active tone in the mesenteric artery was produced by continuous perfusion of 7 μM methoxamine (α₁-adrenoceptor agonist) in the presence of 5 μM guanethidine, which was added to block adrenergic neurotransmission. In this preparation, injections of ACh (1 nmol) and SNP (10 nmol) caused a sharp decrease in perfusion pressure due to vasodilation (Fig. 5A), indicating that the vascular endothelium and smooth muscle remained intact. PNS at 1, 2, 4, and 8 Hz caused a frequency-dependent and long-lasting decrease in perfusion pressure due to vasodilation. Injections of CGRP (5 and 10 pmol) also induced concentration-dependent long-lasting vasodilator responses.

To assess neurogenic responses, PNS was applied in the presence of 0.5 μM tetrodotoxin. As shown in Figs. 5B and 6A, tetrodotoxin significantly inhibited the PNS-induced vasodilator responses, but had no effect on vasodilator responses to CGRP (Fig. 6B), ACh (Fig. 6C), and SNP (Fig. 6D).

Effect of capsaicin on vasodilator responses to PNS and injections of ACh, SNP, and CGRP

As shown in Fig. 7A, in preparations treated with capsaicin and having active tone, vasodilator responses to PNS (2, 4, and 8 Hz) was significantly reduced (Fig. 7B), while the vasodilation in response to CGRP (Fig. 7C), ACh (Fig. 7D), or SNP (Fig. 7E) was not affected.

Effect of CGRP8-37 on vasodilator responses to PNS and injections of CGRP

In preparations with active tone, CGRP8-37 (a CGRP-receptor antagonist) treatment significantly reduced the PNS (2 Hz) and CGRP (5 pmol)-induced vasodilator response (Fig. 8A), compared with no treatment (Fig. 8B).
Immunohistochemistry

As shown in Fig. 9B, the immunohistochemical study of the male mouse mesenteric artery showed dense innervation of TH-immunopositive nerve fibers, which formed a network around the artery. Also, the mesenteric vein had dense innervation of TH-immunopositive nerve fibers.
As shown in Fig. 9E, dense CGRP-immunopositive nerve fibers surrounded the mouse mesenteric artery like a network. The density of CGRP-immunopositive nerve fibers was less than that of TH-immunopositive nerve fibers. Also, the male mouse mesenteric vein had CGRP-immunopositive nerve fibers (Fig. 9F), but they were sparser than those of the artery. No TH-like immunoreactive (LI)– and CGRP-LI–immunopositive fibers were found in control preparations without the primary antibody and any drug treatment in which arteries were treated with the secondary antibodies alone as a control for nonspecific staining. Horizontal bars indicate 50 μm.

Fig. 9. Immunohistochemical images showing adrenergic tyrosine hydroxylase (TH)-like immunoreactive (LI)– and CGRPergic CGRP-LI–containing nerve fibers in male mouse mesenteric arteries (B, E) and veins (C, F) and in arteries treated with 6-hydroxydopamine (6-OHDA) (G, I) or capsaicin (H, J). Images A and D show experiments without the primary antibody and any drug treatment in which arteries were treated with the secondary antibodies alone as a control for nonspecific staining. Horizontal bars indicate 50 μm.

Discussion

The present study is the first to demonstrate that the electrical stimulation (PNS) of male mouse mesenteric vascular beds induces adrenergic nerve–mediated vasoconstrictr responses and capsaicin-sensitive CGRPergic nerve–mediated vasodilator response in which CGRP, a potent vasodilator peptide, acts as a vasodilator neurotransmitter. The present findings are in good accordance with previous reports that the PNS of male rat mesenteric vascular beds induced adrenergic nerve–mediated vasoconstriction and CGRPergic nerve–mediated vasodilatation (4, 9). In the study of vascular responses with perfused mesenteric vascular beds, male rat preparations are commonly used with few reports on responses in mouse preparations. In mouse studies, the rate of perfusion ranged from 0.4 to 3.5 mL/min (17–19). In the present study, an increase in perfusion rate from 1 to 5 mL/min resulted in an elevation in perfusion pressure in a flow rate–dependent manner due to increased vascular resistance (Fig. 1). Since the elevated perfusion pressure was not affected by the potent vasodilator papaverine, it seems likely that the flow rate–dependent increase results from an increase in vascular resistance, not tone generation. Also, it would seem that the perfused mesenteric vasculature is fully dilated at rest. However, a flow rate of more than 4 mL/min caused spontaneous fluctuations of perfusion pressure, which occurred suddenly in the absence of stimuli. Furthermore, the increase in flow caused an augmentation of PNS-induced vasoconstrictor responses, but a rate of more than 3 mL/min did not induce any further change (Fig. 1). Thus, it is very likely that 3 mL/min is the optimal flow rate for perfusing male mouse mesenteric vascular beds.

The present study showed that PNS of male mouse mesenteric vascular beds with resting tension induced a frequency-dependent vasoconstrictor response, which was abolished by tetrodotoxin, a voltage-gated sodium channel blocker and inhibitor of neurotransmitters (20). It appears that the PNS-induced vasoconstrictor response is neurogenic in nature. Furthermore, the adrenergic neuron blocker guanethidine, which blunts the neurogenic release of the adrenergic neurotransmitter norepinephrine (13), abolished the PNS-induced vasoconstrictor response, suggesting that the sympathetic adrenergic nerve mediates the neurogenic vasoconstriction. Furthermore, the present immunohistochemical study demonstrated male mouse mesenteric arteries and veins to be densely innervated by adrenergic TH-immunopositive nerve fibers, which were sensitive to the adrenergic toxin 6-OHDA, but not capsaicin (Fig. 9), suggesting that sympathetic adrenergic nerves innervate the male mouse mesenteric perivascular nerves, like in male rat mesen-
teric arteries. These findings obtained from male mouse mesenteric vascular beds are in good accordance with previous reports in male rat mesenteric vascular beds (4, 13, 15, 21). It is also likely that TH-immunopositive nerves innervating the small arteries are responsible for the neurogenic vasoconstrictor response.

In the study using male rat mesenteric arteries, pulse duration for field-stimulation has been used at 100 μs (small arteries) (12) to 1 ms (vascular beds) (4, 13). In the present study, the frequency-dependent vasoconstrictor responses to PNS with 30-μs pulse duration were abolished by tetrodotoxin and guanethidine. However, tetrodotoxin or guanethidine could not abolish vasoconstrictor responses to PNS at 50-μs pulse duration. Some minor non-frequency–dependent vasoconstriction was observed in the presence of tetrodotoxin (Fig. 2E). These results suggest that PNS with pulse duration of more than 50 μs stimulates directly vascular smooth muscle to cause vasoconstriction, which is resistant to tetrodotoxin. Thus, it is likely that 30 μs is the optimal pulse duration for the PNS of male mouse perfused mesenteric vascular beds.

The present study demonstrated that the α1-adrenoceptor antagonist prazosin markedly inhibited both PNS- and norepinephrine-induced vasoconstriction, but did not completely abolish either response. It is widely accepted that norepinephrine is the neurotransmitter for sympathetic adrenergic nerves and activates α1- and α2-adrenoceptors. In the male mouse mesenteric artery, but not vein, norepinephrine has been shown to cause vasoconstriction via activation through mainly postsynaptic α1-adrenoceptors (22). Therefore, it is likely that the PNS-induced vasoconstrictor response is mainly mediated by postsynaptic α1-adrenoceptors, which are activated by norepinephrine released from adrenergic nerves. However, prazosin did not abolish the PNS-induced vasoconstriction in the mouse mesenteric artery, suggesting involvement of postsynaptic α2-adrenoceptors. The sympathetic adrenergic nerves have been shown to have the cotransmitter neuropeptide Y and ATP together with norepinephrine in vesicles of nerve endings and release these transmitters via an exocytotic process (2, 12). Therefore, it is assumed that PNS-released neuropeptide Y and ATP and postsynaptic α2-adrenoceptors are involved in the prazosin-resistant vasoconstrictor response to PNS in the male mouse mesenteric artery.

Immunohistochemical and vascular response studies have revealed the distribution and function of CGRPergic nerves in male rat mesenteric vascular beds (4). The PNS of male rat preparations with active tone caused vasodilation, which was abolished by tetrodotoxin, capsaicin, and the CGRP-receptor antagonist CGRP8-37. Recent pharmacological and immunohistochemical studies demonstrated the distribution and vasodilator activity of CGRPergic nerves in mouse mesenteric arteries (23, 24). In the present study, the PNS of male mouse mesenteric vascular beds with active tone produced a frequency-dependent vasodilation, which was abolished by tetrodotoxin and capsaicin treatment. Additionally, injections of CGRP induced a long-lasting vasodilation, which mimicked the response to PNS. Furthermore, both the PNS- and CGRP-induced vasodilator responses were significantly inhibited by CGRP8-37 (Fig. 8). These findings strongly suggest that CGRPergic nerves mediate the PNS-induced vasodilation and CGRP receptors exist in male mouse mesenteric vascular beds. However, CGRP8-37 did not abolish the PNS-induced vasodilation. Since our previous study reported that the vasodilator activity to PNS was almost completely inhibited by pre-treatment of CGRP8-37 at 1 μM in the rat mesenteric arteries (25), it seems likely that there might be different sensitivity to CGRP8-37 between rat and mouse preparation. Furthermore, in the present study, capsaicin treatment did not abolish the PNS-induced vasodilation (Fig. 7). Therefore, it seems likely that the PNS-induced vasodilation is mediated by mainly CGRPergic nerves and partially by other NANC nerves including nitroergic nerves and unknown nerves. This notion is supported by the present findings of dense innervation of CGRP-immunopositive nerve fibers in the arteries, sensitive to capsaicin but not 6-OHDA, along with adrenergic TH-immunopositive nerve fibers. Conversely, the mouse mesenteric artery at rest, without active tone, did not cause vasodilation. This is because the isolated mesenteric artery at rest is fully relaxed and had no autonomic adrenergic outflow to generate active tone. Therefore, the vasoactive tone would be needed to observe vasodilation.

In the present experiments, frequencies lower than 8 Hz caused little or no adrenergic nerve–induced vasoconstriction. On the other hand, CGRPergic nerves–mediated vasodilation was induced by frequencies less than 8 Hz. In the experiments on vasoconstrictor responses to PNS, both adrenergic and CGRPergic nerves were simultaneously stimulated by PNS. In the experiments using male rat preparations, when the function of CGRPergic nerves were eliminated by capsaicin, the adrenergic nerve–mediated vasoconstriction was markedly augmented (9). Therefore, it appears that stimulation at low frequencies predominantly induces CGRPergic nerve–mediated vasodilation to attenuate adrenergic vasoconstriction. In the experiments with CGRPergic nerves, guanethidine was added to block adrenergic neurotransmission. This method makes it easy to observe neurogenic vasodilation. Additionally, vasoconstrictor responses to PNS were studied in preparations at resting tension without active tone. At resting tension, the arteries were fully relaxed. Therefore, stronger stimulation...
(high frequency) might be needed to cause vasoconstriction in preparations with resting tension.

In conclusion, the present findings suggest that the male mouse mesenteric resistance arteries are densely innervated by adrenergic vasoconstrictor nerves and CGRPergic vasodilator nerves. They also suggest that both nerves play a critical role in the neural regulation of mesenteric vascular tone. In the preliminary study using Apo E knockout mice, the results showed that the function of CGRPergic nerves in Apo E knockout mice on a high-fat diet decreased with ageing, suggesting that the hyperlipidemia affects the neurogenic control of vascular tone. Therefore, the present study that determined basic conditions for perfusion and electrical field stimulation in male mouse preparations of mesenteric vascular beds gives useful information for investigating the functions of perivascular nerves in a pathological state using genetic engineering in mice.

References


