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INNERVATION AND FUNCTIONAL CHANGES IN MESENTERIC PERIVASCULAR CALCITONIN GENE-RELATED PEPTIDE- AND NEUROPEPTIDE Y-CONTAINING NERVES FOLLOWING TOPICAL PHENOL TREATMENT

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Abstract—We have previously shown that age-related reduction of innervation and function in mesenteric perivascular calcitonin gene-related peptide-containing vasodilator nerves takes place in spontaneously hypertensive rats. The present study was performed to investigate innervation and functional changes in perivascular calcitonin gene-related peptide- and adrenergic neuropeptide Y-containing nerves after topical treatment with phenol, which damages nerve fibers, around the rat superior mesenteric artery. Under pentobarbital-Na anesthesia, 8-week-old Wistar rats underwent *in vivo* topical application of phenol (10% phenol in 90% ethanol) or saline (sham rats) to the superior mesenteric artery proximal to the bifurcation of the abdominal aorta. After the treatment, the animals were subjected to immunohistochemistry of the 3rd branch of small arteries proximal to the intestine and to vascular responsiveness testing on day 3 through day 14. The innervation levels of calcitonin gene-related peptide-like immunoreactivity containing fibers and neuropeptide Y-like immunoreactivity containing fibers were markedly reduced on day 3 to day 14 and on day 5 to day 14 after the treatment, compared with those in sham-operated rats, respectively. In perfused mesenteric vascular beds isolated from phenol-treated rats, adrenergic nerve-mediated vasoconstriction and calcitonin gene-related peptide nerve-mediated vasodilation in response to periarterial nerve stimulation (2–12 Hz) were significantly decreased on day 3 and day 7. Neurogenic release of norepinephrine in phenol-treated rats on day 7 was significantly smaller that that in sham-operated rats. Nerve growth factor content in the mesenteric arteries of phenol-treated rats was significantly lower than that in sham-operated rats. Administration of nerve growth factor using osmotic mini-pumps for 7 days after the phenol treatment resulted in greater density of calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity fibers than in phenol-treated rats and restored decreased vascular responses to periarterial nerve stimulation. These results suggest that topical phenol-treatment of the mesenteric artery effectively induces functional denervation of perivascular nerves, which can be prevented or reversed by nerve growth factor treatment.

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Abbreviations: CGRP, calcitonin gene-related peptide; LI, like immunoreactivity; NE, norepinephrine; NGF, nerve growth factor; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PNS, periarterial nerve stimulation; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat.

Key words: calcitonin gene-related peptide-containing vasodilator nerves, neuropeptide Y-containing vasoconstrictor nerves, phenol-induced nerve injury, nerve growth factor, rat mesenteric resistance artery.

It has been demonstrated that rat mesenteric resistance arteries are densely innervated by both sympathetic adrenergic nerves and capsaicin-sensitive sensory nerves, which mainly possess calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerve fibers (Kawasaki et al., 1988). Our previous reports presented evidence that CGRPergic nerves suppress adrenergic nerve-mediated vasoconstriction via CGRP release, and conversely, adrenergic nerves presynaptically inhibit the neurogenic release of CGRP from the nerve to decrease CGRPergic nerve function (Kawasaki et al., 1990a). Thus, we have proposed that CGRPergic vasodilator nerves and sympathetic vasoconstrictor nerves reciprocally regulate the tone of the mesenteric resistance artery. Also, we have reported that the density of CGRPergic vasodilator nerve innervation in the mesenteric resistance artery of spontaneously hypertensive rats (SHR) decreases with ageing and suggested that the remodeling of the CGRPergic innervation contributes to the development and maintenance of hypertension in SHR (Kawasaki et al., 1990b, 1992, 2001). Furthermore, we have demonstrated that angiotensin II type-1 receptor (AT1R) antagonist and angiotensin converting enzyme (ACE) inhibitor have the ability to attenuate the decreases in both the innervation of CGRP-containing nerves and the expression of CGRP mRNA in SHR (Kawasaki et al., 1999, 2001, 2003; Hobara et al., 2005). Thus, we proposed that prevention and reversal of the remodeling of CGRPergic nerve innervation may be therapeutic for hypertension.

Some reports have shown that peripheral nerves such as the chorda tympani, lingual nerves (Smith et al., 2004) and motor neurons in hypoglossal nerves (Okura et al., 1999) are able to regenerate new processes if they are supplied with growth-promoting substrates. However, there have been no studies on re-innervation and /or redistribution of perivascular nerves, including sympathetic adrenergic nerves and CGRPergic nerves.

Therefore, the present study was designed to establish an animal model for perivascular nerve remodeling with re-innervation and/or redistribution of adrenergic nerves and CGRPergic nerves by using topical treatment with phenol, which has been used to block peripheral nerve

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activity (Wang and Bukoski, 1999). The innervation density and vascular responses of the rat mesenteric resistance artery after perivascular nerve damage with phenol treatment were investigated in this study. Furthermore, we examined whether nerve growth factor (NGF) is able to exert a protective effect on perivascular nerve innervation in the phenol-treated model.

EXPERIMENTAL PROCEDURES

Experimental animals

Eight-week-old Wistar rats (purchased from Shimizu Experimental Animals, Shizuoka, Japan) were used in this study. The 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 with a 12-h light/dark cycle (lights on at 8:00 AM). This study was performed in accordance with the Guideline for Animal Experiments, at Okayama University. Every effort was made to minimize the number of animals used and their suffering.

Surgical procedures

Under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), an abdominal midline incision was made in the animals, and the superior mesenteric artery proximal to bifurcation from the abdominal aorta was carefully exposed and topically painted with 10% phenol solution (in 90% alcohol–saline) using a cotton bud. To examine the influence of the operation, sham-operated rats underwent the same surgical procedures but with painting with vehicle (saline or 90% alcohol without including phenol) instead of phenol solution. After the painting, an antibiotic (penicillin G) was infused around the surgical area and then the incision was closed. After the operation, the animal was transferred into the individual cage in the temperature-controlled room and received i.m. injection of an antibiotic for three consecutive days. After phenol treatment and sham operation, the animals were killed by deep anesthesia for use in the experiments described below on day 3, day 5, day 7, day 10 and day 14.

Systolic blood pressure (SBP) measurement

The SBP of each animal was measured daily by tail-cuff plethysmography (model TK-370C; UNICOM, Tokyo, Japan) throughout the treatment period.

Immunohistochemical study

The animals treated with phenol and saline were anesthetized with a large dose of sodium pentobarbital (100 mg/kg, intraperitoneally). The superior mesenteric artery was cannulated with polyethylene tubing and infused with Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) and the mesenteric artery was removed together with the intestine as described previously (Hobara et al., 2005). The third branch of the mesenteric artery proximal to the intestine was removed and immersion-fixed in the Zamboni solution for 48 h. After fixation, the artery was placed in a microtube (1 ml) and repeatedly rinsed in phosphate-buffered saline (PBS) and immersed in PBS containing 0.5% Triton X-100 overnight, and then incubated with PBS containing normal goat serum (1:100) for 60 min. The artery was then incubated with the rabbit polyclonal anti-CGRP (Biogenesis Ltd., Poole, UK) antiserum at the dilution of 1:300 or the rabbit polyclonal anti-neuropeptide Y (NPY) (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) antiserum at the dilution of 1:300 for 72 h at 4 °C. After the incubation, the artery was washed in PBS and the site of the antigen–antibody reaction was revealed by incubation with fluorescein-5-isothiocyanate (FITC)-labeled goat anti-rabbit IgG (diluted 1:100) (ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 60 min. Thereafter, the arteries were thoroughly washed in PBS, mounted on slides and coverslipped with glycerol/PBS (2:1 v/v) and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss GmbH, Jena, Germany) in Okayama University Medical School Central Research Laboratory.

Immunocytochemical analysis

The immunostaining density of CGRP-like immunoreactive (CGRP-LI) and NPY-LI nerve fibers was analyzed with the method as described by Hobara et al. (2005). Since the fluorescence intensity differed depending on the day of the experiment, the mesenteric arteries from both phenol-treated rats on day 0, day 3, day 5, day 7, day 10 and day 14 and day-matched sham rats were isolated, fixed, and immunostained at the same time on the same day and mounted on the same slide glass, and non-treated rats at day 0 were used as a control for the intensity in each experiment. For the quantitative evaluation of NPY-LI and CGRP-LI, confocal projection images of NPY and CGRP immunostaining, which were patched together with eight to 10 overlapping images (0.1 μm scanning), were magnified at 20× and digitized as TIF images using a digital camera system (Olympus SP-1000, Olympus, Tokyo, Japan) and imported into a Windows XP computer (Toshiba, Tokyo, Japan). The stored digital images were analyzed using image-processing software (Simple PCI; Compix Inc., Imaging Systems, Cranberry Township, PA, USA). The extraction of specific color and measured field commands were used to extract the NPY-LI and CGRP-LI areas (which were stained green). Extraction of the signal was carried out using specific protocols based on the hue, lightness and saturation color parameter. A measured field of 100 μm×100 μm (10,000 μm^2), which contained the adventitia layer including immunostained perivascular nerve fibers, was randomly selected on magnified images of the whole mount artery. The objective areas command was used to calculate the percentage of NPY-LI- and CGRP-LI-positive area, and the intensity of staining was estimated using a point counting computer program and the background level was subtracted from the experimental value to yield the corrected intensity. The average of the density in three arteries was taken as the nerve density per animal.

To determine the number of CGRP-LI and NPY-LI fibers, five horizontal lines were drawn on the image of the blood vessel in the same region where the density was estimated by computer analysis. Then, the number of fibers that crossed each line was counted and the average of the number in three arteries was taken as the total number of fibers per animal.

Perfusion of mesenteric vascular beds

The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and the mesenteric vascular bed was isolated and prepared for perfusion as described previously (Kawasaki et al., 1988, 1990c). The superior mesenteric artery was cannulated and flushed gently with a modified (see below) Krebs–Ringer bicarbonate solution (Krebs solution) to eliminate blood in 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 the 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 5 ml/min with a peristaltic pump (model AC-2120, ATTO Co., Tokyo, Japan). The preparation was also 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% O_2 plus 5% CO_2 before passage through a warming coil maintained at 37 °C.

The modified Krebs solution had the following composition (mM): NaCl 119.0, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, EDTA–2Na 0.03, and glucose 11.1 (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T, Nihon Kohden, Tokyo, Japan) and recorded using a pen recorder (model U-228, Nippon Denshi Kagaku, Tokyo, Japan).

Perivascular nerve stimulation (PNS) and bolus injection of norepinephrine (NE) or CGRP

After allowing the basal perfusion pressure to stabilize, the preparation was initially subjected to PNS at 8 and 12 Hz and bolus injections of NE (5 and 10 nmol; Daiichi-Sankyo, Tokyo, Japan), and then was contracted with an alpha1-adrenoceptor agonist, methoxamine (7 μM; Nihon Shinyaku Co., Kyoto, Japan), in the presence of an adrenergic neuron blocker, guanethidine (5 μM; Tokyo Kasei, Tokyo, Japan), which was added to block the adrenergic neurotransmission. The increased perfusion pressure was allowed to stabilize, and the preparation was again subjected to PNS at 1, 2 and 4 Hz and bolus injection of CGRP (25, 50 and 100 pmol; Peptide Institute, Osaka, Japan). PNS was applied by using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and supramaximal voltage (50 V) were given for 30 s using an electronic stimulator (model SEN 3301, Nihon Kohden). NE and CGRP were directly injected into the perfusate proximal to the arterial cannula with an infusion pump. A volume of 100 μl was injected for 12 s.

At the end of each experiment, the preparations were perfused with 100 μM papaverine (Sigma Aldrich Japan) to cause complete relaxation. Vasodilator activity is expressed as the percentage of the perfusion pressure at maximum relaxation induced by papaverine. Vasoconstrictor activity is expressed as the percentage of the perfusion pressure.

Measurement of NE in the perfusate

Under pentobarbital anesthesia, mesenteric vascular beds isolated from phenol- and saline-treated rats were prepared for perfusion as described above. In preparations with resting tone, the perfusate was collected before and after PNS (8 and 12 Hz) for 3 min. NE in the perfusate was adsorbed onto alumina and eluted with acetic acid. The eluate was assayed by high-performance liquid chromatography (HPLC) with electrochemical detection

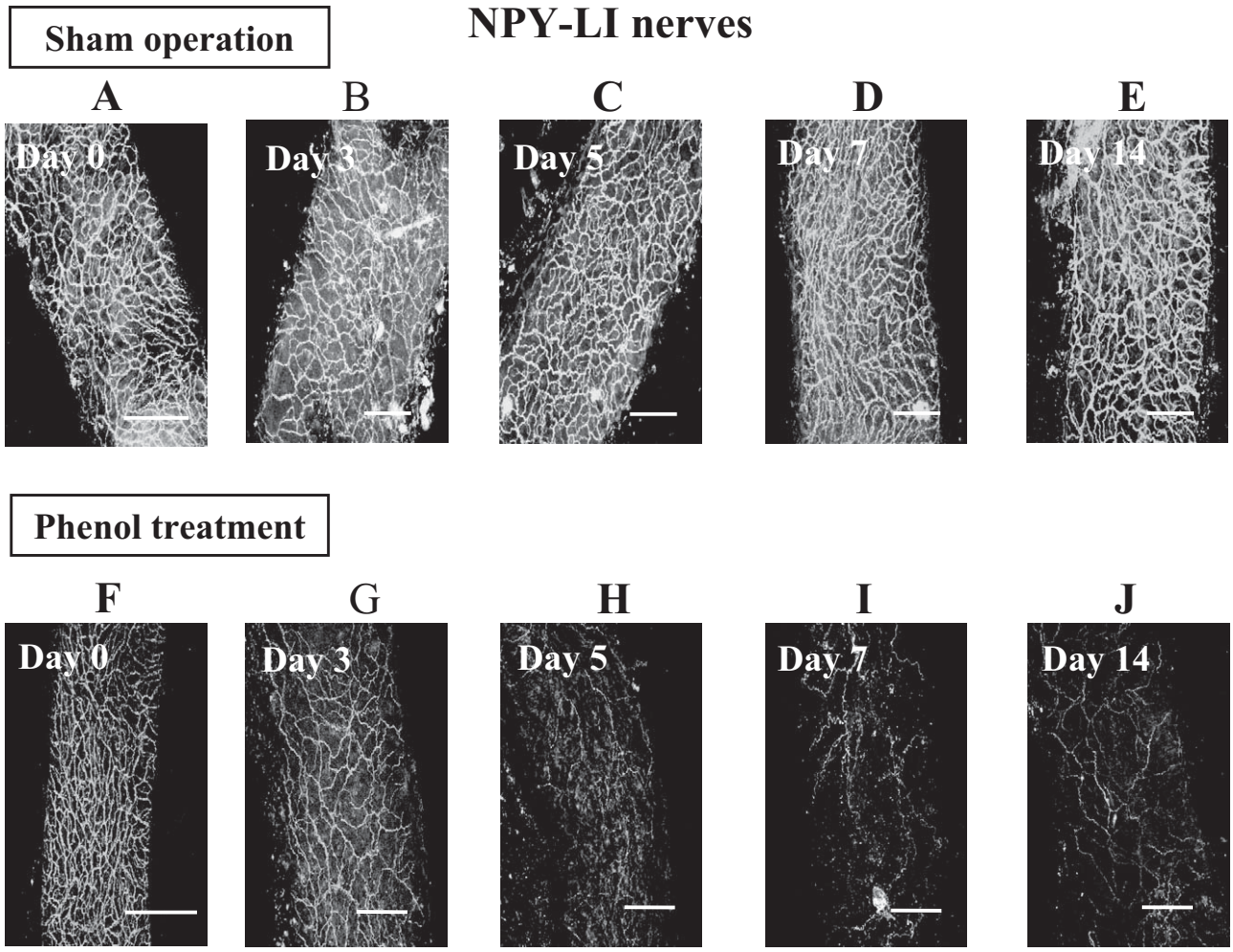

Fig. 1. Representative confocal laser micrographs showing changes in the density of NPY-LI nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery. Upper images (A, B, C, D and E) and lower images (F, G, H, I and J) show arteries isolated from sham-operated rats and phenol-treated rats, respectively. The images of A and F indicate intact arteries before the treatment. The images of B and G, C and H, D and I, and E and J indicate day 3, day 5, day 7 and day 14 after the treatment, respectively. The scale bar=100 μm in the right lower corner of each panel.

(model HTEC-500, Eicom, Kyoto, Japan). The internal standard was dihydroxybenzylamine.

Measurement of NGF contents in the mesenteric artery

The animals were anesthetized with pentobarbital-Na (50 mg/kg, i.p.) and killed by decapitation. The mesenteric arteries of the first to second branches were carefully isolated and minced with scissors. Then, the mesenteric arteries were homogenized with homogenizer in cold homogenization buffer consisting of 100 mM Tris–HCl, pH 7.0, containing 2% bovine serum albumin (BSA), 1 M NaCl, 0.1% NaN_3, 4 mM EDTA, 2% Triton X-100. Homogenates were prepared in 50 volumes of homogenization buffer relative to tissue wet weight. The homogenates were centrifuged at 14,000×*g* for 30 min at 4 °C and the supernatant was used for the NGF assay using an NGF kit, which utilized a sandwich enzyme immunoassay (EIA) (Chemicon Inc., Temecula, CA, USA). In the NGF assay system, sheep polyclonal antibodies generated against mouse NGF were coated onto a microplate and used to capture NGF from the sample. NGF-specific mouse monoclonal antibodies detected the captured NGF. Peroxidase-labeled mouse-specific donkey polyclonal antibodies were used to determine the amount of NGF. The protein concentration of the solution was determined by the ultraviolet absorption method.

NGF administration

A mini-osmotic pump (model 2001, Alzet; Alza, Palo Alto, CA, USA) containing NGF (Sigma Aldrich Japan) or saline was s.c. implanted in the abdominal area immediately after the phenol-painting surgery, and NGF was intraperitoneally administered at a rate of 20 μg/kg/day for a period of 7 days. NGF, which was dissolved in sterile saline, or sterile saline was injected into an Alzet osmotic mini pump. Control animals were implanted with the mini-pump containing saline.

Statistical analysis

All data were expressed as mean±S.E.M. Analysis of variance (ANOVA) followed by the Spearman's *t*-test and Tukey's test was used to determine statistical significance where appropriate. Correlation analysis was carried out by using Pearson's correlation test. A value of $P<0.05$ was considered statistically significant.

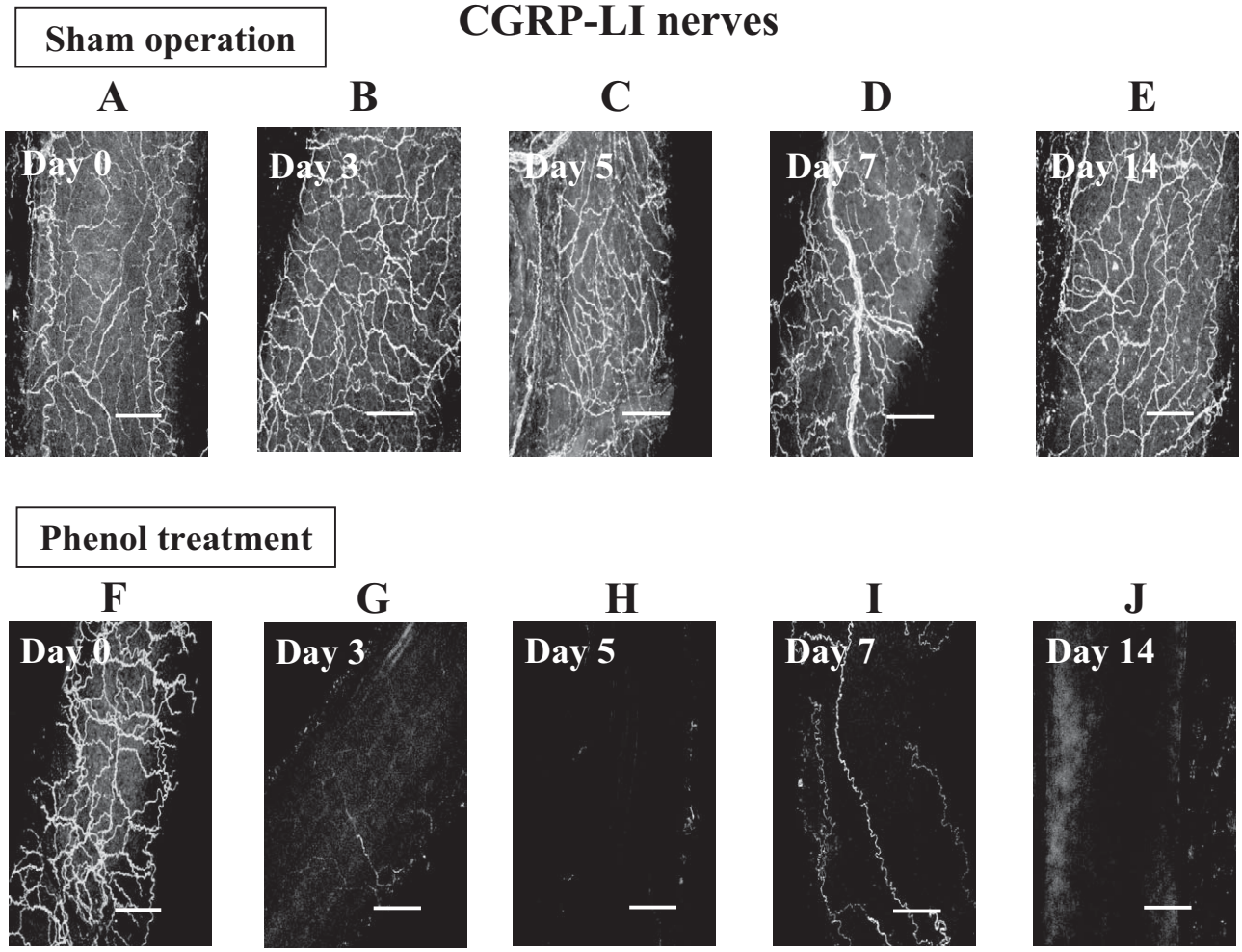

Fig. 2. Representative confocal laser micrographs showing changes in the density of CGRP-LI-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery. Upper images (A, B, C, D and E) and lower images (F, G, H, I and J) show arteries isolated from sham-operated rats and phenol-treated rats, respectively. The images of A and F indicate intact arteries before the treatment. The images of B and G, C and H, D and I, and E and J indicate day 3, day 5, day 7 and day 14 after the treatment, respectively. The scale bar=100 μm in the right lower corner of each panel.

RESULTS

Changes in innervation of CGRP- or NPY-LI nerve fibers in mesenteric arteries following phenol treatment

Topical application of phenol solution to the superior mesenteric artery caused a marked reduction of innervation of NPY-LI nerve fibers and CGRP-LI nerve fibers in the distal small artery, whereas saline or vehicle treatment did not reduce the innervation of either of these types of nerve fibers. Figs. 1 and 2 show typical patterns of the time-courses of changes in the innervation of NPY-LI and CGRP-LI nerve fibers in small mesenteric arteries after topical application of phenol or vehicle (sham rats). To quantitatively evaluate the time-courses of the density of NPY-LI and CGRP-LI nerve fibers in the mesenteric artery, an immunocytochemical technique was used in this study. Different time-courses of the density of NPY-LI and CGRP-LI nerve fibers were observed, as shown in Fig. 3. The density of NPY-LI fibers decreased approximately 30% at 3 days and 40% at 5 and 7 days after the phenol application and maximally decreased by approximately 50% at 14 days after the application. In contrast, the density of CGRP-LI fibers was markedly reduced by approximately 70% at 3 days and maximally 80% at 5 days after the phenol application and the reduced density level was maintained until 14 days after the application (Fig. 3).

The relationships between the numbers of CGRP-LI and NPY-LI nerve fibers, which were visually counted, and the densities of CGRP-LI and NPY-LI nerves (%), which were quantified by computer-assisted image processing, were assessed in the mesenteric arteries of the phenol-treated rats. There were significant positive correlations between the density and the numbers of CGRP-LI and NPY-LI nerve fibers at 3 days (CGRP-LI, $P<0.01$, $r=0.960$; NPY-LI, $P<0.01$, $r=0.7192$), 5 days (CGRP-LI, $P<0.01$, $r=0.847$; NPY-LI, $P=0.21$, $r=0.327$), 7 days (CGRP-LI, $P<0.01$, $r=0.822$; NPY-LI, $P<0.01$, $r=0.740$), 10 days (CGRP-LI, $P<0.01$, $r=0.654$; NPY-LI, $P<0.01$, $r=0.871$) and 14 days (CGRP-LI, $P<0.01$ $r=0.918$; NPY-LI, $P<0.01$, $r=0.706$) after phenol application (data not shown).

Changes in SBP after phenol treatment

As shown in Fig. 4, the SBP at 3, 5 and 7 days after phenol treatment was significantly lower than that in the sham group (saline treatment).

Adrenergic nerve-mediated vasoconstrictor response in phenol-treated, sham-operated rats

As shown in Fig. 5, PNS at 8 and 12 Hz of the perfused mesenteric vascular beds with resting tone produced a frequency-dependent increase in perfusion pressure due to vasoconstriction. The PNS-induced vasoconstriction was abolished by alpha1-adrenoceptor antagonist (prazosin) and adrenergic neuron blocker (guanethidine) (data not shown), indicating that the response is mediated by NE released from the stimulation of periarterial adrenergic nerves. Bolus injections of NE at concentrations of 5 and 10 nmol also caused concentration-dependent vasoconstriction, which was blocked by prazosin but not guanethidine (data not shown), indicating that the response was mediated by the stimulation of postsynaptic alpha1-adrenoceptors.

As shown in Figs. 5 and 6, the vasoconstrictor responses to PNS at 8 and 12 Hz in phenol-treated rats on day 3 were significantly smaller than those in day-matched sham-operated rats (Figs. 5 and 6). Furthermore, in phenol-treated rats on day 7, the vasoconstrictor response to

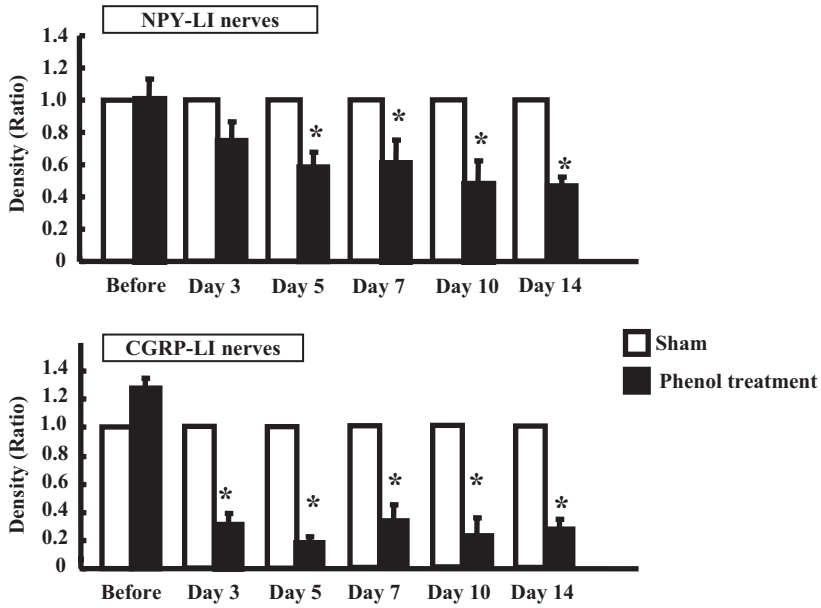

Fig. 3. Bar graphs showing changes in the density of NPY-LI- and CGRP-LI-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery. The vertical scale indicates the fold increase over the value in the sham control. Each bar indicates mean±S.E.M. of five to eight experiments. * $P<0.05$ vs. sham control.

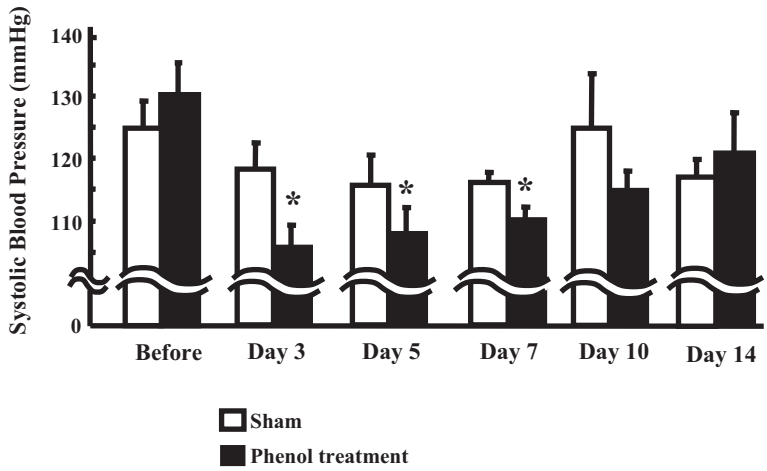

Fig. 4. Changes in SBP after topical phenol treatment of the superior mesenteric artery. Each point indicates the mean±S.E.M. of five to eight experiments. * $P<0.05$ vs. sham control.

PNS at 12 Hz but not 8 Hz was also significantly smaller than that in day-matched sham-operated rats (Fig. 6). As shown in Figs. 5 and 6, vasoconstrictor responses to bolus injections of NE were not significantly altered after phenol or sham treatment. There was no significant difference between phenol and sham treatment.

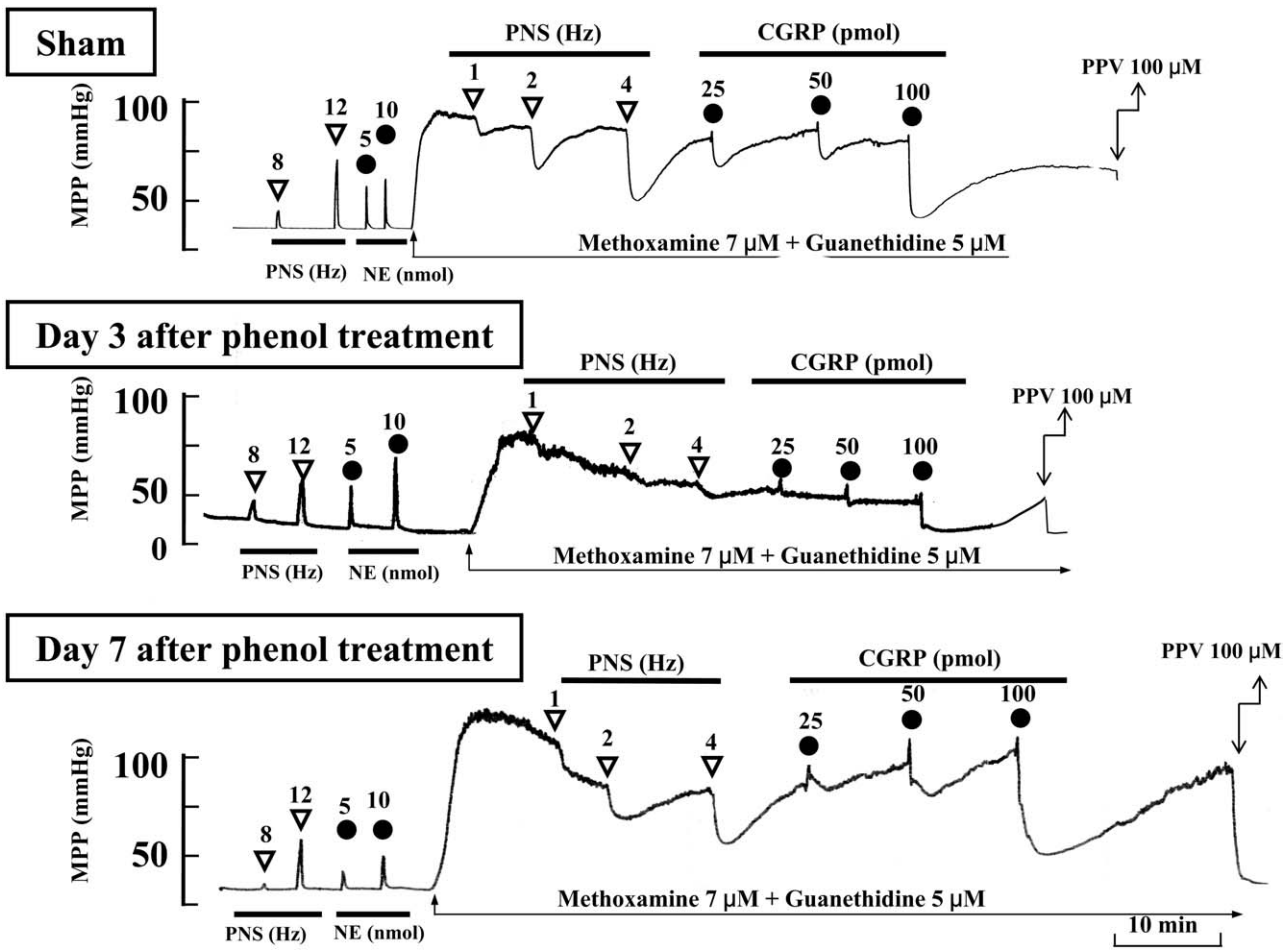

Fig. 5. Typical records showing changes in vasoconstrictor and vasodilator responses to PNS and to bolus infusions of NE (5 and 10 nmol) and rat CGRP (25, 50 and 100 pmol) in perfused mesenteric vascular beds after topical phenol treatment of the superior mesenteric artery. Active tone of the preparation was produced by 7 μM methoxamine in the presence of 5 μM guanethidine. MPP, mean perfusion pressure; PPV, papaverine perfusion.

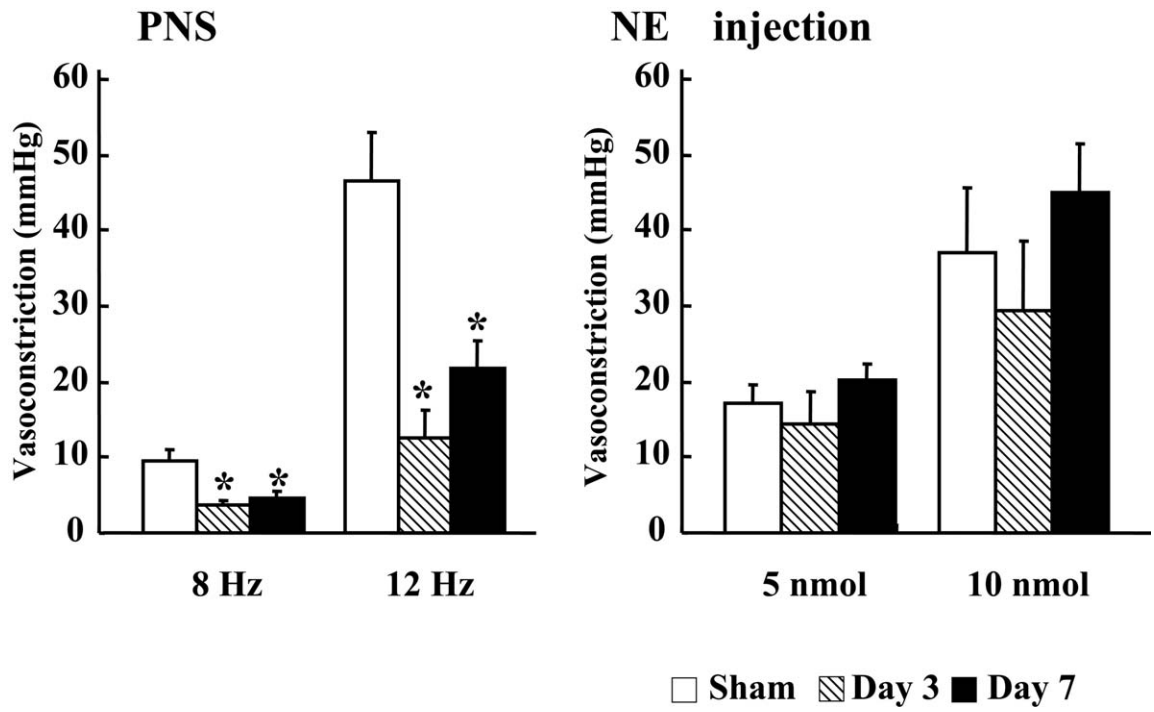

Fig. 6. Bar graphs showing changes in vasoconstrictor responses to PNS and to bolus infusion of NE in perfused mesenteric vascular beds after topical phenol treatment of the superior mesenteric artery. * $P<0.05$ vs. sham control.

Neurogenic vasodilator response in phenol-treated, sham-operated rats

To maintain the active tone of the mesenteric artery, the preparation was contracted by continuous perfusion of 7 μM methoxamine (alpha1-adrenergic receptor agonist) in the presence of 5 μM guanethidine (adrenergic neuron blocker), which was added to block adrenergic neurotransmission. In this preparation, PNS at 1, 2 and 4 Hz caused a frequency-dependent decrease in perfusion pressure due to vasodilation. The vasodilator response to PNS has been shown to be mediated by CGRPergic nerves, since the response is blocked by CGRP receptor antagonist [CGRP(8–37)] and CGRP depletor (capsaicin) (Han et al., 1990; Kawasaki and Takasaki, 1992). Bolus injections of CGRP also induced concentration-dependent vasodilation, which has been shown to be mediated by postsynaptic CGRP receptors (Kawasaki et al., 1990b).

As shown in Fig. 7, there was no significant difference in the methoxamine-induced rises in mean perfusion pressure before PNS between phenol- and sham-operated rats at any of the times examined after treatment. The PNS-induced (1 and 2 Hz) vasodilator responses of preparations on day 3 were similar in magnitude to those in day-matched sham preparations, but vasodilation by 4 Hz PNS on day 3 and 2 and 4 Hz PNS on day 7 was significantly smaller than that in day-matched sham rat (Fig. 7). However, no significant difference in CGRP-induced vasodilation was found between phenol- and sham-treated rats.

NE concentrations in the perfusate

In the perfused mesenteric vascular beds, NE was detected in the perfusate before PNS. PNS of mesenteric vascular beds at 8 and 12 Hz evoked a frequency-dependent increase in the NE concentration of the perfusate (Table 1). The PNS-evoked NE increase has been shown to be due to the release of NE from vascular adrenergic nerves, since the release is blocked by guanethidine (adrenergic neuron blocker) and tetrodotoxin (neurotoxin) (Westfall et al., 1987).

As shown in Table 1, the spontaneous release of NE before PNS was reduced by phenol treatment. Additionally, the PNS (8 and 12 Hz) -evoked NE release on day 7 after phenol treatment was significantly smaller than that in day-matched sham rats.

NGF concentrations in the mesenteric artery

NGF was detected in the small branch of the rat mesenteric artery. The content of NGF was significantly reduced on day 7 after phenol treatment compared with that in the day-matched sham-operated group (Table 2).

Effect of NGF on changes in innervation of CGRP- or NPY-LI nerve fibers in mesenteric arteries and vascular responses of perfused mesenteric vascular beds following phenol treatment

The density of NPY-LI nerve fibers decreased approximately 50% at 7 days after the phenol application. The reduction of NPY-LI fiber density following phenol treatment was not observed in NGF-treated rats. The density of

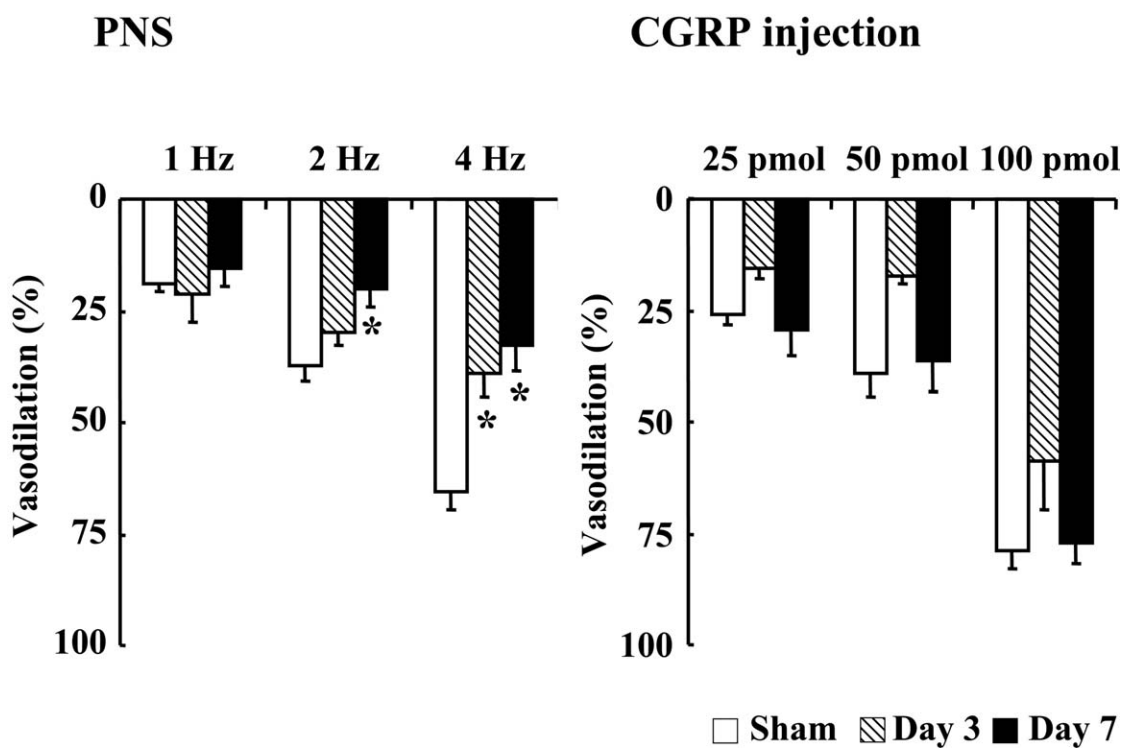

Fig. 7. Bar graphs showing changes in vasodilator responses to PNS and to bolus infusion of CGRP in perfused mesenteric vascular beds after topical phenol treatment of the superior mesenteric artery. Active tone of the preparation was produced by 7 μM methoxamine in the presence of 5 μM guanethidine. Relaxation is expressed as a percentage of the maximum relaxation induced by perfusion of 100 μM papaverine at the end of each experiment. * $P<0.05$ vs. sham control.

NPY-LI nerve fibers in NGF-treated arteries was significantly greater than that in saline-treated arteries, and similar to that in the sham arteries (Fig. 8).

As shown in Fig. 9, topical treatment of the superior mesenteric artery with phenol caused approximately an 80% reduction of the density of CGRP-LI nerve fibers in distal small mesenteric arteries, while administration of NGF after the phenol treatment did not cause a significant decrease in the density. The density of CGRP-LI nerve fibers in NGF-treated arteries was significantly greater than that in saline-treated arteries and almost the same as that in the sham arteries.

The relationships between the numbers of CGRP-LI and NPY-LI nerve fibers, which were visually counted, and the densities of CGRP-LI and NPY-LI nerves (%), which were quantified by computer-assisted image processing, were assessed in mesenteric arteries of all groups. There were significant positive correlations between the density and the numbers of CGRP-LI and NPY-LI nerve fibers in the sham (CGRP-LI, $P<0.01$, $r=0.585$; NPY-LI, $P<0.01$, $r=0.762$), phenol (CGRP-LI, $P<0.01$, $r=0.822$; NPY-LI, $P<0.01$, $r=0.740$), and NGF (CGRP-LI, $P<0.01$ $r=0.715$; NPY-LI, $P=0.317$, $r=0.235$) groups (data not shown).

As shown in Fig. 10A, in NGF-treated preparations, adrenergic nerve-mediated vasoconstrictor responses to PNS at 8 and 12 Hz were greater than those in saline-treated preparations, while vasoconstrictor responses to bolus injections of NE did not change significantly after NGF treatment.

As shown in Fig. 10B, CGRPergic nerve-mediated vasodilator responses to PNS (1 Hz) of preparations treated with saline or NGF were similar in magnitude to those in sham

Table 1. Changes in neurogenic release of NE induced by PNS in perfused mesenteric vascular beds on day 7 after topical phenol treatment of the superior mesenteric artery

Treatment	PNS (8 Hz)			PNS (12 Hz)		
	Pre-PNS pg/ml	Post-PNS pg/ml	Net release pg/ml	Pre-PNS pg/ml	post-PNS pg/ml	Net release pg/ml
Sham ($n=6$)	35.3±11.2	57.6±12.7	22.3±5.8	39.3±21.7	75.0±24.3	35.7±9.8
Day 7 ($n=8$)	31.2±21.6	40.5±5.3	9.3±2.3*	26.9±13.1	44.3±7.1	17.4±5.7*

Data are expressed as means±S.E.M. NE concentration is expressed as pg/ml. Pre and post indicate NE concentrations before PNS and after PNS, respectively. Net release indicates post-PNS NE minus pre-PNS NE.
* $P<0.05$ vs. sham control.

Table 2. NGF content in the small mesenteric artery at 7 days after topical phenol treatment of the superior mesenteric artery

Treatment	NGF (pg/pg total protein)
Sham (n=6)	26.5±7.6
Phenol (n=6)	8.6±2.3*

Values indicate mean±S.E.M.
* $P<0.05$ vs. sham control.

preparations. The vasodilation in response to PNS in NGF-treated preparations was slightly but not significantly greater than that in saline-treated preparations. The vasodilator response to bolus injection of CGRP at a concentration of 100 pmol in NGF-treated preparations was significantly smaller than that in the sham or saline-treated preparations.

DISCUSSION

The present findings demonstrated that topical treatment of the large superior mesenteric artery with phenol caused a marked reduction of perivascular nerve innervation in the distal small branch of the mesenteric artery. Treatment with phenol resulted in a 70% reduction of CGRP-LI nerves and 40% reduction of NPY-LI nerves 7 days later, compared with those in sham-operated rats, suggesting that phenol treatment causes non-selective damage to the perivascular nerves, both sympathetic adrenergic nerves and CGRPergic nerves. The density of CGRP-LI nerve fibers was almost nil on day 3 after phenol treatment and this phenomenon lasted until day 14, while the density of NPY-LI nerves was gradually decreased after the treatment. These findings suggest that vascular CGRPergic nerves are more sensitive to this treatment. Chang et al. (2004) reported that injuring somatic and visceral neurons in the hypoglossal nucleus decreased CGRP-immunoreactivity at 7 days after axotomy, while choline acetyltransferase-IR (i.e. cholinergic nerves) was markedly reduced at 3 days. Therefore, it is possible that degeneration of CGRP-LI nerves and NPY-LI nerves in the rat mesenteric artery may occur through different processes.

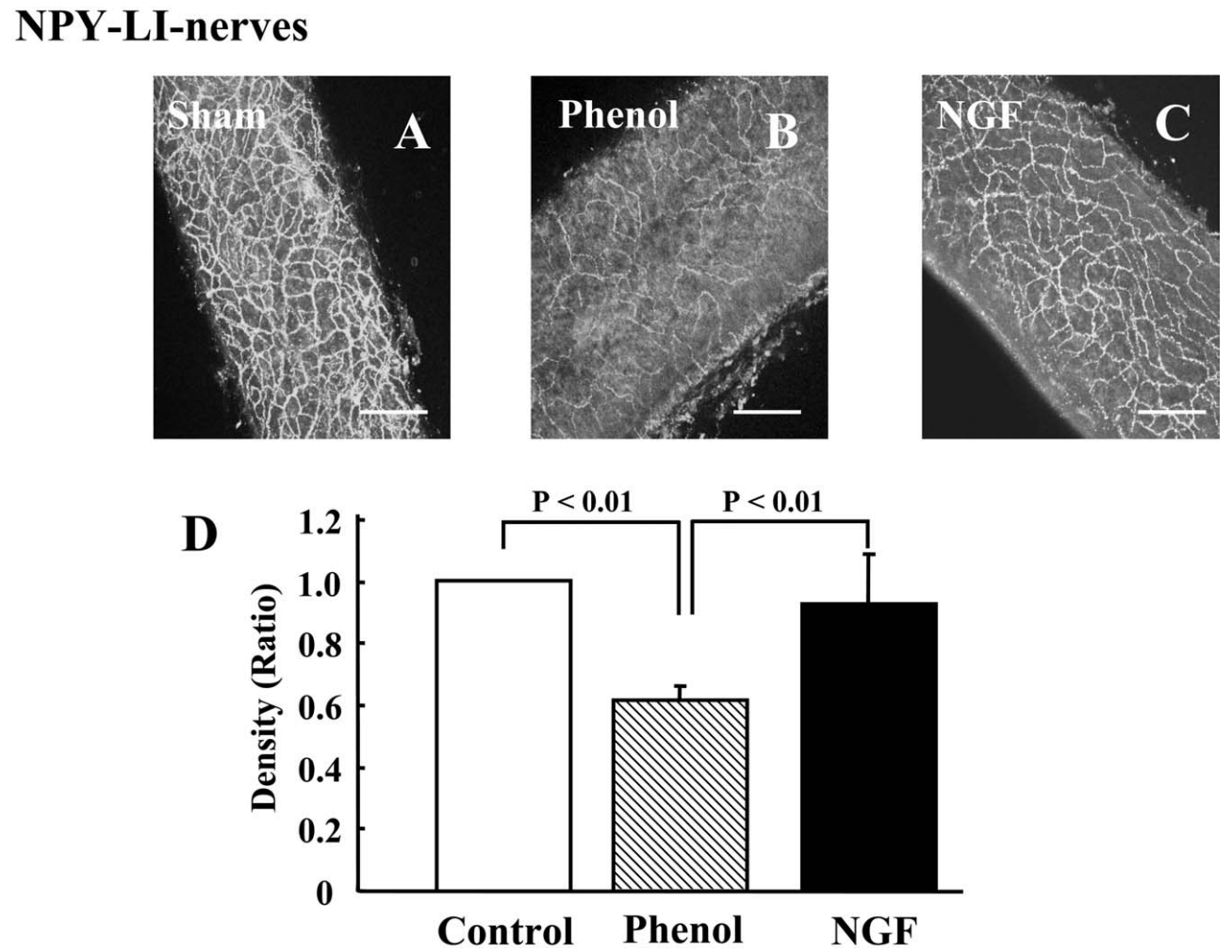

Fig. 8. Typical images (A, B, C) and bar graph (D) showing the effect of NGF administration for 7 days on the density of NPY-LI-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery. (A) Sham preparation. (B) Saline/phenol-treated preparation. (C) NGF/phenol-treated preparation. The scale bar=100 μm in the right lower corner of each image. (D) The vertical scale indicates the fold increase over the value in the sham control.

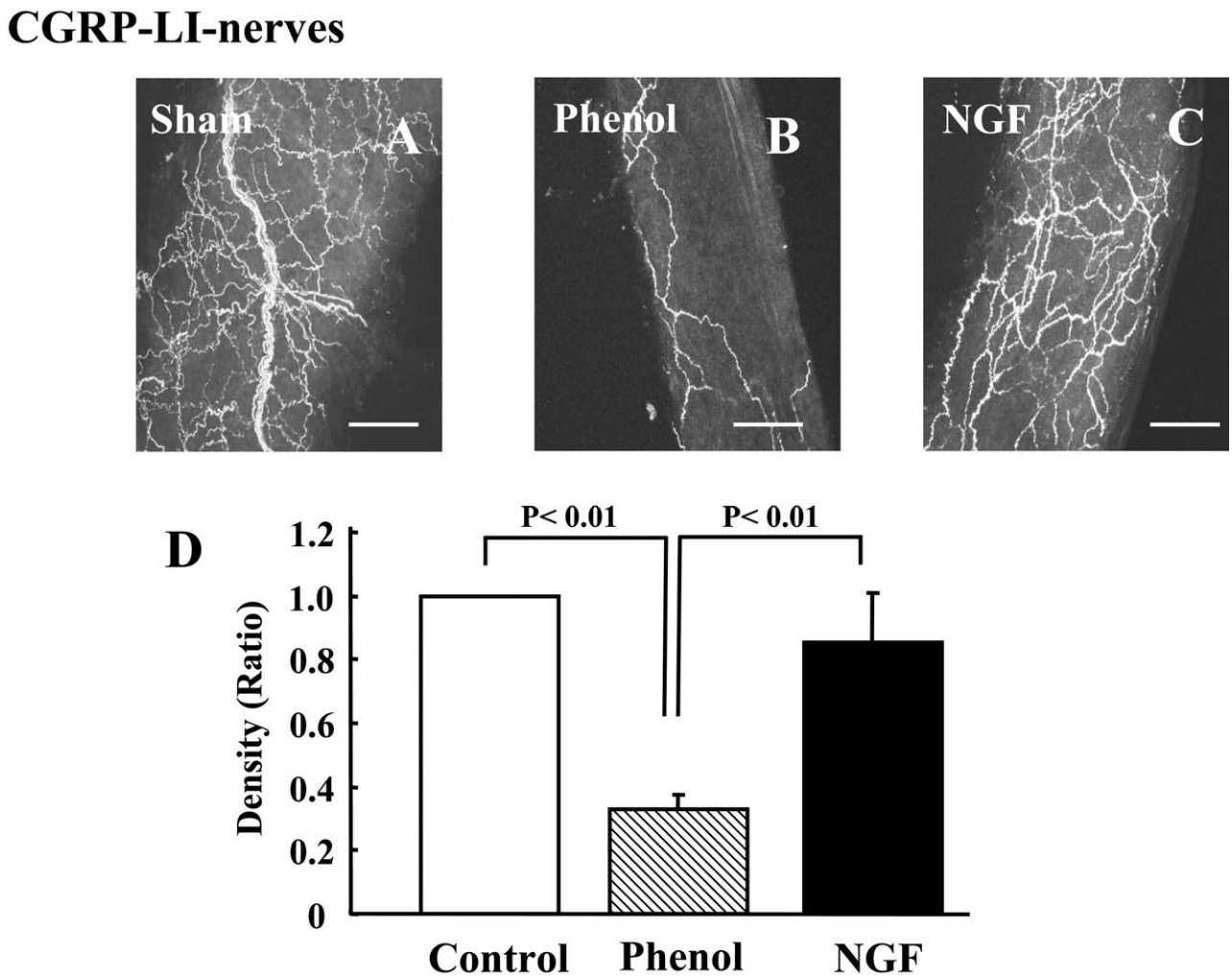

Fig. 9. Typical images (A, B, C) and bar graph (D) showing the effect of NGF administration for 7 days on the density of CGRP-LI-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery. (A) Sham preparation. (B) Saline/phenol-treated preparation. (C) NGF/phenol-treated preparation. The scale bar=100 μm in the right lower corner of each image. (D) The vertical scale indicates the fold increase over the value in the sham control.

In the present study, a significant fall in SBP was observed 3, 5 and 7 days after the treatment with phenol, compared with the SBP in sham-operated rats. The time-course of the decrease of SBP was similar to that of the reduction of the density of both CGRP- and NPY-LI nerves, suggesting that the reduction of mesenteric perivascular nerve innervation is responsible for the SBP decrease. Sensory nerve-denervation of rats with capsaicin has been reported to result in no significant difference in SBP compared with that in control rats, whereas significantly higher SBP than the control SBP has been shown in high sodium diet-induced hypertensive rats with sensory nerve-denervation with capsaicin (Wang et al., 1998, 2001). In those studies, capsaicin was administered systemically to selectively destroy capsaicin-sensitive sensory nerves in the whole body. Therefore, it is unclear whether destruction of capsaicin-sensitive vascular nerves, including CGRPergic nerves, was responsible for the alteration of SBP. In contrast, the present findings that topical treatment of the superior mesenteric artery with phenol resulted in a marked reduction of CGRP- and NPY-LI containing nerves and a fall in SBP suggest that the mesenteric perivascular nervous system plays an important role in blood pressure regulation.

The neuropeptide NPY has been shown to be synthesized in the sympathetic ganglion cells and axonally transported to and stored in postganglionic sympathetic nerve varicosities, where it colocalizes with NE (Ekblad et al., 1984; Fried et al., 1986; Lundberg, 1996). It is well known that the adrenergic transmitter NE is synthesized in the adrenergic nerve varicosities, but its synthase is axonally transported to the varicosities from the sympathetic ganglion cells. This implies that the NPY-LI-containing nerves are the postganglionic sympathetic nerves. Treatment of the postganglionic nerve trunk innervating the mesenteric artery with phenol resulted in the destruction of the NPY-LI-containing nerve fibers, and led to a significant decrease in the neurogenic release of NE induced by PNS. Therefore, it is likely that phenol treatment damaged the nerve axons or inter-

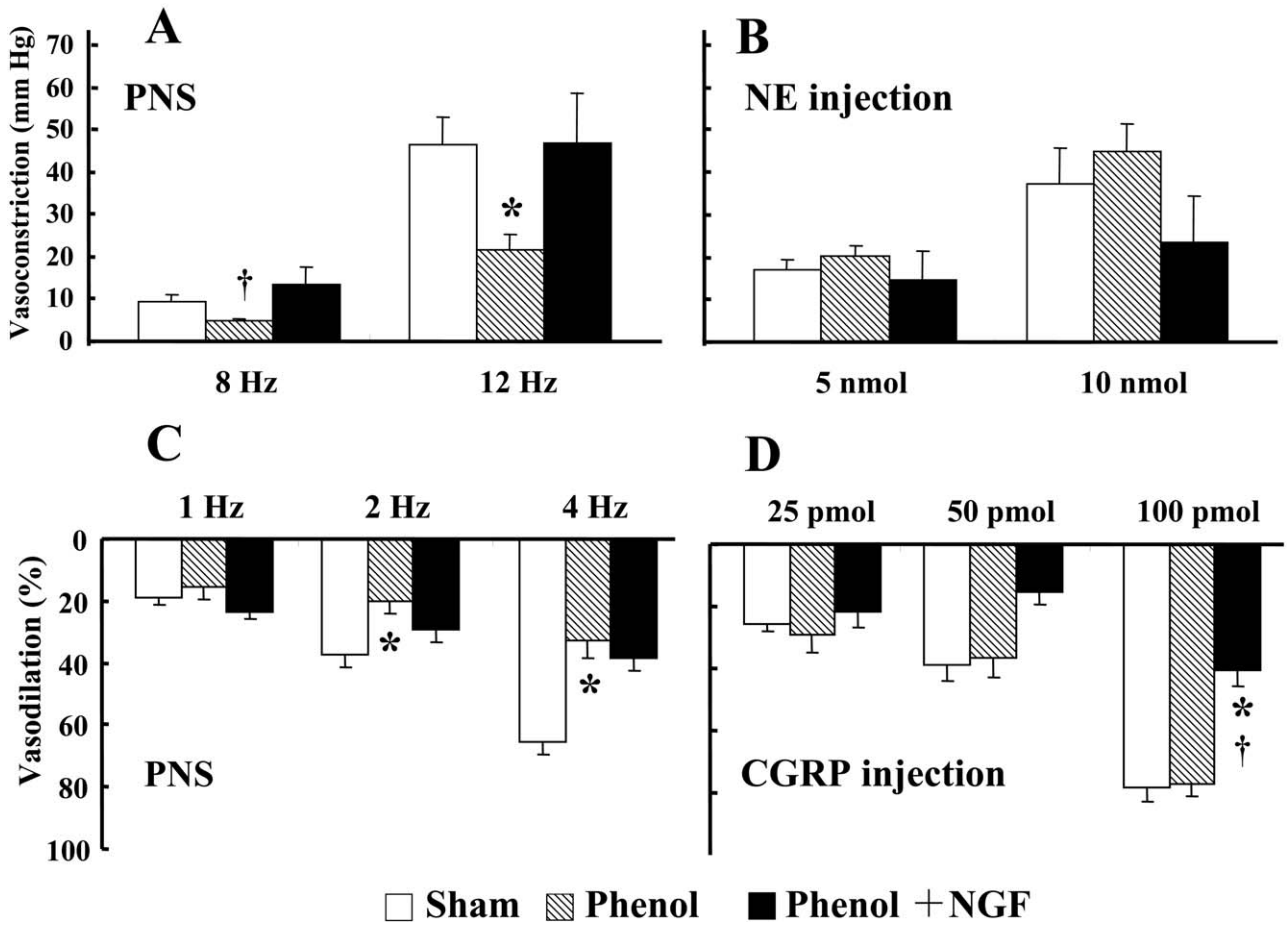

Fig. 10. Effect of NGF administration for 7 days on vasoconstrictor responses to PNS (A) and bolus injection of NE (B) and vasodilator responses to PNS (C) and bolus injection of CGRP (D) in perfused mesenteric vascular beds after topical phenol treatment of the superior mesenteric artery. * $P<0.05$ vs. sham control. † $P<0.05$ vs. phenol treatment.

rupted the axonal transportation of transmitter synthase enzyme and NPY to nerve terminals.

Westerlund et al. (2001), who studied the morphological effects of phenol–glycerol treatment, reported that sciatic nerves were dissolved 1–2 weeks after intraneural injection of 7% phenol, which led to the induction of hemorrhagic necrosis, and then the nerve fibers were dissolved and empty basal lamina tubes were expanded but did not collapse (Westerlund et al., 2003). Furthermore, protein gene product 9.5 immunoreactivity (PGP-9.5-ir), which is a specific marker for nerve tissue, has been shown to be absent in the mesenteric artery treated with phenol (Liu et al., 1996). These reports suggest that topical phenol treatment might damage nerve trunks around the treated region to cause degeneration of distal nerve terminals, which results in a decrease of NPY-LI nerve innervation and neurogenic NE release.

In the present study, neither vasoconstrictor responses to exogenously applied NE nor vasodilator responses to exogenous CGRP injection were altered by phenol treatment, which greatly reduced the neurogenic vasoconstrictor and vasodilator response to PNS. These findings suggest that the sensitivities of postsynaptic receptors may not be affected by short-term denervation of perivascular nerves. In a study of spinal cord transection for 15 or 30 days, alpha1- and alpha2-adrenoceptors were shown to increase in cat lumbar segments (Giroux et al., 1999). An increase in the density of alpha1-adrenoceptors has been reported in the spinal cord after adrenergic denervation by 6-hydroxydopamine (Roudet et al., 1993). Xie et al. (2001) reported the up-regulated expression of alpha1b-adrenoceptor mRNA but not alpha1a- or alpha1d-adrenoceptor mRNA in the dorsal root ganglia after spinal nerve ligation. These studies suggest the possibility that nerve destruction in the CNS may increase the sensitivity of postsynaptic alpha-adrenoceptors. Injuring sciatic sensory neurons has been shown to increase the sensitivity of alpha-adrenoceptors but not beta-adrenoceptors (Korenman and Devor, 1981; Devor et al., 1994). However, it is unlikely that denervation in the perivascular nervous system causes changes in the sensitivity of postsynaptic alpha-adrenoceptor changes, although further investigations are needed to test this directly.

NGF was the first protein discovered in the family of neurotrophins (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1987). NGF is produced in tissues innervated by neurons and transported from nerve endings to cell bodies and is an autocrine factor for sympathetic nerves (Hasan et al., 2003). Additionally, a recent study sug-

gested that the sympathetic neurons express NGF mRNA and synthesize and secrete pro-NGF protein (Hasan et al., 2003). NGF levels of target tissues have been shown to correlate with the density of sympathetic innervation of tissues (Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). The present study demonstrated a significant decrease in NGF content after phenol treatment of the mesenteric artery, suggesting the reduced density of perivascular nerves. This finding is in good agreement with an earlier study suggesting that axotomy induced the loss of target-derived factors such as NGF (Nja and Purves, 1978). Therefore, the present findings that administration of NGF resulted in an increase in the density of both CGRP-LI and NPY-LI nerve fibers after phenol application suggest that mesenteric perivascular nerves could regenerate and/or reinnervate tissues. It has been demonstrated that s.c. administration of NGF markedly increases CGRP release from rat dorsal horn (Bowles et al., 2004). Furthermore, intraventricular administration of brain-derived neurotrophic factor (BDNF) increases NPY and substance P expression in brain of the newborn rat (Nawa et al., 1994). These reports suggest that exogenously administered NGF can facilitate release of neuropeptides such as CGRP and NPY. This notion may explain the present finding that administration of NGF restored vasoconstriction mediated by stimulation of perivascular adrenergic nerves. However, exogenous NGF had less effect on restoration of CGRP-nerve mediated vasodilation. Therefore, it is likely that functional restoration induced by NGF is mainly due to regeneration and/or reinnervation of perivascular nerves.

CONCLUSION

In summary, the present study showed that topical phenol treatment of the superior mesenteric artery induced destruction of periarterial nerves innervating the distal artery and functional loss of perivascular nerve-mediated vascular responses. Furthermore, the present findings that administration of NGF facilitated reinnervation of perivascular nerves after phenol application suggest that perivascular nerves have an ability to undergo regeneration and/or reinnervation. This model will provide a novel experimental paradigm for investigating the function and regeneration of perivascular nerves.

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