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Nerve growth factor facilitates redistribution of adrenergic and non-adrenergic non-cholinergic perivascular nerves injured by phenol in rat mesenteric resistance arteries

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ABSTRACT

We previously reported that nerve growth factor (NGF) facilitated perivascular sympathetic neuropeptide Y (NPY)- and calcitonin gene-related peptide (CGRP)-containing nerves injured by the topical application of phenol in the rat mesenteric artery. We also demonstrated that mesenteric arterial nerves were distributed into tyrosine hydroxylase (TH)-, substance P (SP)-, and neuronal nitric oxide synthase (nNOS)-containing nerves, which had axo-axonal interactions. In the present study, we examined the effects of NGF on phenol-injured perivascular nerves, including TH-, NPY-, nNOS-, CGRP-, and SP-containing nerves, in rat mesenteric arteries in more detail. Wistar rats underwent the *in vivo* topical application of 10% phenol to the superior mesenteric artery, proximal to the abdominal aorta, under pentobarbital-Na anesthesia. The distribution of perivascular nerves in the mesenteric arteries of the 2nd to 3rd-order branches isolated from 8-week-old Wistar rats was investigated immunohistochemically using antibodies against TH-, NPY-, nNOS-, CGRP-, and SP-containing nerves. The topical phenol treatment markedly reduced the density of all nerves in these arteries. The administration of NGF at a dose of 20 μg/kg/day with an osmotic pump for 7 days significantly increased the density of all perivascular nerves over that of sham control levels. These results suggest that NGF facilitates the reinnervation of all perivascular nerves injured by phenol in small resistance arteries.

1. Introduction

Perivascular adrenergic nerves innervating resistance arteries play an important role in maintaining vascular tone and regulating organ and tissue blood flow. Resistance vessels, such as mesenteric arteries, are also innervated by non-adrenergic non-cholinergic (NANC) nerves including calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves (Kawasaki et al., 1988) and nitric oxide (NO)-containing nerves (Hatanaka et al., 2006). We previously reported that adrenergic and CGRPergic nerves interacted reciprocally in order to regulate vascular tone (Kawasaki et al., 1990). We also demonstrated that adrenergic and NO-containing nerves innervating rat mesenteric arteries were involved in the modulation of adrenergic neurotransmission (Hatanaka et al., 2006). These findings propose that perivascular adrenergic and NANC nerves innervating mesenteric arteries play an important role in regulating vascular tone via axo-axonal interactions. CGRP and substance P (SP) as neuropeptides have been shown to colocalize in the same sensory neuron. However, CGRP-like immunoreactive (LI) and SP-LI nerves have been shown to have

Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; LI, like immunoreactive; NANC, non-adrenergic non-cholinergic nerves; NGF, nerve growth factor; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; PBS, phosphate-buffered saline; p75NTR, neurotrophin receptor; SP, substance P; TH, tyrosine hydroxylase; TrkA, tropomyosin receptor kinase A

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different distribution patterns and neuromodulatory roles in nociception and peripheral cardiovascular responses in several species (Skofitsch and Jacobowitz, 1985; Kawasaki et al., 1988; Tsuda and Matsuyama, 1991; Henderson et al., 2006). Therefore, the function of SP-LI nerves in mesenteric arteries has not yet been elucidated. Tyrosine hydroxylase (TH) is a rate-limiting enzyme for noradrenaline synthase and is contained in sympathetic adrenergic nerves, which act as vasoconstrictor nerves. TH-LI nerves have been shown to coexist with neuropeptide Y (NPY)-LI nerves in rat mesenteric arteries and skeletal muscle arteries of various kinds of animals, including rabbits, dogs, cats, and guinea pigs (Pernow et al., 1987; Smyth et al., 2000; Gradin et al., 2003).

We previously reported that the *in vivo* topical application of phenol, which has been used to block peripheral nerve activity (Wang and Bukoski, 1999), on superior mesenteric arteries markedly reduced the distribution of sympathetic adrenergic NPY-containing nerves and CGRPergic nerves in rat mesenteric resistance arteries. Furthermore, we showed that nerve growth factor (NGF) facilitated the reinnervation of both types of nerves injured by phenol (Hobara et al., 2006). Previous studies found that adrenomedullin (a vasodilator peptide), angiotensin II (a vasoconstrictor peptide), and hepatic growth factor facilitated the reinnervation of mesenteric perivascular nerves injured by topical treatments with phenol (Hobara et al., 2005, 2007a, 2007b, 2008). These findings revealed that facilitatory effects of angiotensin II via the stimulation of angiotensin II type 2 receptors and hepatic growth factor on the reinnervation of mesenteric perivascular nerves were selective to CGRPergic nerves (Hobara et al., 2007b, 2008), thereby implying that the phenol-induced lesion of perivascular nerves is useful for identifying substances with neurotrophic effects. However, it currently remains unknown whether NGF facilities the reinnervation of TH-, neuronal nitric oxide synthase (nNOS)-, and SP-containing nerves injured by the application of phenol.

Therefore, we herein investigated facilitatory effects of NGF on the reinnervation of phenol-injured perivascular nerves including adrenergic NPY- and TH-containing nerves, as well as CGRPergic nerves, nNOS-, and SP-containing nerves innervating rat mesenteric resistance arteries.

2. Materials and methods

2.1. Experimental animals

Eight-week-old Wistar rats were used in this study and purchased from Shimizu Laboratory Supplies Co, Ltd. (Kyoto, Japan). 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 AM). This study was carried out to minimize the number of animals used and suffering, and was performed 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).

2.2. In vivo phenol treatment

The *in vivo* phenol treatment used to injure perivascular nerves innervating the mesenteric arteries of the rat was performed according to the method reported by Hobara et al. (2006). Under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), an abdominal midline incision was made in the rat, and the superior mesenteric artery proximal to bifurcation from the abdominal aorta was carefully exposed and topically swabbed with 10% phenol solution (in 90% alcohol–saline) using a cotton bud. After swabbing, an antibiotic (penicillin G; Sigma-Aldrich Japan, Tokyo, Japan) was infused around the surgical area, and the incision was then closed. In order to examine the influence of this surgery, sham-operated rats underwent same procedures, but were swabbed with a vehicle (saline or 90% alcohol without including phenol) instead of the phenol solution. After surgery, animals were moved into individual cages in a temperature-controlled room, and received intramuscular injections of penicillin G (3.1 mg/kg) for 3 consecutive days. Seven days after the phenol treatment and sham operation, animals were killed by deep anesthesia for use in experiments described below.

2.3. Administration of NGF

NGF was administered intraperitoneally by a continuous infusion through a mini-osmotic pump (model 1007D, Alzet; Alza, Palp Alto, CA, USA) for 7 days. A mini-pump was implanted into the abdominal area immediately after the phenol-swabbing surgery, and NGF was administered at a rate of 20 μg/kg/day according to Hobara et al. (2006). NGF was dissolved in sterile saline and injected into the osmotic mini-pump.

2.4. Immunohistochemical study

A polyethylene tubing cannula was inserted into the superior mesenteric artery under anesthesia with pentobarbital-Na (50 mg/kg, intraperitoneally), phosphate-buffered saline (PBS) was perfused to remove blood in the vascular bed, and Zamboni solution was then infused. The mesenteric vascular bed was removed together with the intestine as described previously (Hobara et al., 2005, 2006). The 2nd (250–300 μm in diameter) and 3rd branches (200–250 μm in diameter) of the mesenteric artery proximal to the intestine were removed and immersion-fixed in Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) for 48 h. After fixation, each artery was repeatedly rinsed in PBS, immersed in PBS containing 0.5% Triton X-100 overnight, and incubated with PBS containing normal goat serum (1: 100) for 60 min. The tissue was then incubated with rabbit polyclonal anti-TH serum (1: 500) (Chemicon International, Inc., Temecula, CA, USA), rabbit anti-NPY serum (1: 300) (Phoenix Pharmaceuticals Inc., Belmont, CA, USA), rabbit polyclonal anti-CGRP serum (Biomol GmbH, Hamburg, Germany) (1: 500), anti-SP serum (1: 200) (Biomol GmbH), or anti-nNOS serum (1: 500) (Zymed Laboratories, South San Francisco, CA, USA) at 4 °C for 72 h. After being incubated, arteries were washed in PBS and sites of the antigen-antibody reaction were revealed by incubation with fluorescein-5-isothiocyanate-labeled goat anti-rabbit IgG (diluted 1: 100) (ICN Pharmaceuticals, Inc., Orange, CA, USA) for 60 min. Thereafter, the artery was thoroughly washed in PBS, mounted on slides, cover-slipped with glycerol/PBS (2: 1 v/v), and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss GmbH, Jena, Germany) in the Okayama University Medical School Central Research Laboratory.

2.5. Immunohistochemical analysis

The immunostaining density of TH- LI, NPY-LI, nNOS-LI, CGRP-LI, and SP-LI nerves was analyzed with the method described by Hobara et al. (2005, 2006). Since the fluorescence intensity differed depending on the day of the experiment, the 2nd and 3rd branches of mesenteric arteries were isolated, fixed, and immunostained at the same time on the same day, and were mounted on the same slide glass. Mesenteric arteries isolated from sham-operated rats were used as a control for intensity in each