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ADRENOMEDULLIN FACILITATES REINNERVATION OF PHENOL-INJURED PERIVASCULAR NERVES IN THE RAT MESENTERIC RESISTANCE ARTERY

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Abstract—Our previous report showed that innervation of calcitonin gene-related peptide (CGRP)- and neuropeptide Y (NPY)-containing nerves in rat mesenteric resistance arteries was markedly reduced by topical application of phenol, and that nerve growth factor (NGF) facilitates the reinnervation of both nerves. We also demonstrated that a CGRP superfamily peptide, adrenomedullin, is distributed in perivascular nerves of rat mesenteric resistance arteries. In the present study, we investigated the influence of adrenomedullin on the reinnervation of mesenteric perivascular nerves following topical phenol treatment. Under pentobarbital-Na anesthesia, 8-week-old Wistar rats underwent *in vivo* topical application of phenol (10% phenol in 90% ethanol) to the superior mesenteric artery proximal to the bifurcation of the abdominal aorta. After the treatment, the animals were subjected to immunohistochemistry of the third branch of small arteries proximal to the intestine and to vascular responsiveness testing on day 7. Topical phenol treatment caused marked reduction of the density of NPY-like immunoreactive (LI)- and CGRP-LI nerve fibers in the arteries. Adrenomedullin (360 or 1000 ng/h) or NGF (250 ng/h), which was administered intraperitoneally for 7 days using an osmotic mini-pump immediately after topical phenol treatment, significantly increased the density of CGRP-LI- and NPY-LI nerve fibers compared with saline. Treatment with adrenomedullin (1000 ng/h) or NGF restored adrenergic nerve-mediated vasoconstriction and CGRP nerve-mediated vasodilation in the perfused mesenteric artery treated topically with phenol. These results suggest that adrenomedullin, like NGF, has a facilitatory effect on the reinnervation of perivascular nerves.

Key words: adrenomedullin, calcitonin gene-related peptide-containing nerves, neuropeptide Y-containing nerves, neurotrophic action, perivascular nerves, rat mesenteric artery.

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Abbreviations: CGRP, calcitonin gene-related peptide; LI, like immunoreactive; mRNA, messenger RNA; NGF, nerve growth factor; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PNS, periarterial nerve stimulation; SHR, spontaneously hypertensive rat.

Adrenomedullin is a 52-amino-acid peptide hormone, originally isolated from human pheochromocytoma (Kitamura et al., 1993) and has been detected in a variety of tissues, including the adrenal medulla, heart, kidney, lung, pancreas and brain (Ichiki et al., 1994; Asada et al., 1999; Serrano et al., 2000). The systemic administration of adrenomedullin in peripheral sites decreases the mean arterial pressure (Khan et al., 1997) and intrarenal infusion of adrenomedullin increases the renal blood flow, glomerular filtration rate and renal sodium excretion (Jougasaki et al., 1995). Additionally, i.v. administration of adrenomedullin decreases pulmonary vascular resistance in primary pulmonary hypertension (Nagaya and Kangawa, 2004). On the other hand, the administration of adrenomedullin into the brain has been shown to inhibit water drinking and salt intake (Murphy and Samson, 1995; Samson and Murphy, 1997) and cause diuretic and natriuretic actions (Israel and Diaz, 2000).

Adrenomedullin has partial sequence homology with calcitonin gene-related peptide (CGRP), which is a potent vasodilator peptide. The main distribution of CGRP in peripheral sites has been shown to be the primary sensory nerves (Lee et al., 1985), and the dorsal root ganglia is a prominent site of CGRP synthesis and contains the cell bodies of the sensory afferent neurons (Marti et al., 1987). We previously reported evidence that the rat mesenteric artery is innervated by nonadrenergic noncholinergic vasodilator nerves in which CGRP acts as a neurotransmitter (Kawasaki et al., 1988, 1998). Furthermore, we suggested that CGRP-containing nerves together with sympathetic adrenergic nerves regulate the tone of the blood vessels (Kawasaki et al., 1991). Adrenomedullin has been shown to act on CGRP receptors to produce vasodilation of the rat mesenteric artery (Nuki et al., 1993; Ishizaka et al., 1994), implying that the peptide may act as a neurotransmitter or neuromodulator in the mesenteric artery. Moreover, our previous study using immunohistochemistry showed dense innervation of adrenomedullin-containing perivascular nerves in the rat mesenteric artery and detected adrenomedullin immunoreactivity and adrenomedullin messenger RNA (mRNA) expression in the rat dorsal root ganglia (Hobara et al., 2004). Our previous report also showed evidence of the colocalization of adrenomedullin and CGRP in the perivascular nerves and dorsal root ganglia (Hobara et al., 2004). Therefore, it is inferred that adrenomedullin, which is colocalized with CGRP in the capsaicin-sensitive nerve, has neuronal activity such as nerve differentiation. However, the role

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of adrenomedullin in the perivascular nerves remains unclear.

The CGRP immunoreactivity and CGRP mRNA expression have been reported to be rapidly up-regulated in the affected motoneurons following peripheral axotomy (Piehl et al., 1998), suggesting that CGRP may be involved in axonal regeneration or may exert trophic influence on the lesioned neurons (Dumoulin et al., 1992). Additionally, adrenomedullin has been demonstrated to have neuroprotective activity in ischemic brain (Dogan et al., 1997; Watanabe et al., 2001; Miyashita et al., 2006) and protecting the blood-brain barrier against oxidative stress (Chena et al., 2005). These studies suggest that adrenomedullin plays some role in the neuronal function. However, it is unclear whether adrenomedullin has a protective effect or is a survival-promoting factor for perivascular nerves.

More recently, we demonstrated that innervation of CGRP- and neuropeptide Y (NPY)-containing nerves in rat mesenteric resistance arteries was markedly reduced by topical application of phenol, and that nerve growth factor (NGF) facilitates reinnervation of both types of nerves (Hobara et al., 2006). Therefore, the present study was designed to test the hypothesis that adrenomedullin has the ability to facilitate reinnervation of mesenteric perivascular nerves after *in vivo* denervation induced by topical treatment with phenol, which has been used to block peripheral nerve activity (Wang and Bukoski, 1999). To investigate the reinnervation of perivascular nerves and their function, the innervation density and neurogenic vascular responses of the rat mesenteric resistance artery after perivascular nerve damage with phenol treatment were examined in this study.

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 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). Every effort was made to minimize the number of animals used and their suffering. All experiments conformed to international guidelines on the ethical use of animals.

Surgical procedures

The *in vivo* denervation of perivascular nerves in the mesenteric arteries of the rats was performed as described previously (Hobara et al., 2006). Briefly, under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), an abdominal midline incision was made, 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 the swabbing, an antibiotic (penicillin G; Sigma Aldrich Japan, Tokyo, Japan) was infused around the surgical area and then the incision was closed. To examine the influence of the operation, sham-operated rats underwent the same surgical procedures but with swabbing with vehicle (saline or 90% alcohol without including phenol) instead of phenol solution. After the operation, the animals were transferred into individual cages in the temperature-controlled room and received intramuscular injection of penicillin G (3.1 mg/kg) for three consecutive days. After phenol treatment and sham operation, the animals were killed by deep anesthesia for use into subject to the experiments described below on day 7.

Adrenomedullin and NGF administration

A mini-osmotic pump (model 2001, Alzet; Alza, Palo Alto, CA, USA) containing human recombinant adrenomedullin (Peptide Institute, Osaka, Japan), NGF (Sigma Aldrich Japan) or saline was implanted in the abdominal area immediately after the phenol-swabbing surgery, and adrenomedullin at a rate of 360 or 1000 ng/h or NGF at a rate of 250 ng/h was administered i.p. for a period of 7 days. Adrenomedullin or NGF was dissolved in sterile saline and injected into an osmotic mini-pump. Control animals were implanted with mini-osmotic pumps containing sterile saline.

Immunohistochemical study

The animals treated with phenol and saline were anesthetized with a large dose of sodium pentobarbital (100 mg/kg, i.p.). 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, and the mesenteric artery was removed together with the intestine as described previously (Hobara et al., 2005, 2006). 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 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 tissue was then incubated with the rabbit polyclonal anti-NPY (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) antiserum at the dilution of 1:300 or the rabbit polyclonal anti-CGRP (Biogenesis Ltd., Poole, UK) antiserum at the dilution of 1:300 for 72 h at 4 °C. After the incubation, the artery was washed in PBS, and the sites of antigen-antibody reaction were revealed by incubation with fluorescein-5-isothiocyanate-labeled goat anti-rabbit IgG (diluted 1: 100) (ICN Pharmaceuticals, Inc., Aurora, OH, 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 Okayama University Medical School Central Research Laboratory.

Immunocytochemical analysis

The immunostaining density of NPY-like immunoreactive (LI) and CGRP-LI nerve fibers was analyzed with the method as described by Hobara et al. (2005, 2006). Since the fluorescence intensity differed depending on the day of the experiment, the mesenteric arteries from both phenol-treated rats on day 7 and saline-treated control rats were isolated, fixed and immunostained at the same time on the same day and mounted on the same slide glass, and the saline-treated rats at day 7 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 8–10 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 100×100 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 areas, 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 NPY-LI and CGRP-LI fibers, 5 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, i.p.) and the mesenteric vascular bed was isolated and prepared for perfusion as described previously (Kawasaki et al., 1988, 1991, 1998). 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 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 or CGRP

After the basal perfusion pressure was allowed to stabilize, the preparation was initially subjected to PNS by electrical stimulation at 8 and 12 Hz and bolus injections of norepinephrine (5 and 10 nmol) (Daiichi-Sankyo Co., Tokyo, Japan), and then was induced to contract with α1-adrenoceptor agonist, methoxamine (7 μM) (Nippon Shinyaku Co., Kyoto, Japan), in the presence of an adrenergic neuron blocker, guanethidine (5 μM) (Sigma Aldrich 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 by electrical stimulation at 1, 2 and 4 Hz and bolus injections of CGRP (25, 50 and 100 pmol) (Peptide Institute). 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). Norepinephrine 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 (Dainippon-Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan) to cause complete relaxation. Vasodilator activity is expressed as percentage of the perfusion pressure at maximum relaxation induced by papaverine. Vasoconstrictor activity is expressed as percentage of the perfusion pressure.

Statistical analysis

All data were expressed as mean±S.E.M. Analysis of variance (ANOVA) followed by Tukey's test was used to determine statistical significance where appropriate. Correlation analysis was carried out using Pearson's correlation test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effects of adrenomedullin and NGF administration on changes in innervation of NPY-LI or CGRP-LI nerve fibers in the mesenteric artery following topical phenol treatment

As shown in Fig. 1A, D, the rat mesenteric artery was densely innervated by both NPY-LI and CGRP-LI nerve fibers, and the density of NPY-LI nerves was much higher than that of CGRP-LI nerves. The topical application of phenol on the superior mesenteric artery markedly reduced the innervation of both NPY-LI (Fig. 1B) and CGRP-LI (Fig. 1E) nerve fibers in the distal small artery, whereas vehicle (saline or 90% alcohol) treatment (sham control) did not reduce the innervation of either type of nerve fibers (Fig. 1A, D). As shown in Fig. 1C, F, NGF administration immediately after topical application of phenol restored the level of innervation of NPY-LI or CGRP-LI nerve fibers to the level observed in the sham-treated control rats.

Figs. 2 and 3 show the effect of adrenomedullin or NGF administration on the phenol-induced decrease in the density of perivascular NPY-LI and CGRP-LI nerves. To quantitatively evaluate changes in the density of NPY-LI and CGRP-LI nerve fibers in the mesenteric artery, an immunocytochemical technique was used in this study. As shown in Fig. 2B, F, the density of NPY-LI nerve fibers decreased approximate 50% at 7 days after topical phenol application on the superior mesenteric artery. There was a significant difference between the density in the phenol-saline-treated and sham-treated control rats. The reduction of the NPY-LI fiber density level following phenol treatment was restored by the application of NGF (250 ng/h) to the level in the sham-treated control rats (Fig. 2E, F). There was a significant difference between the density in the phenol-saline-treated and NGF-treated rats (Fig. 2F).

Treatment with adrenomedullin at a dose of 1000 ng/h but not 360 ng/h restored the density of NPY-LI fibers (Fig. 2D) to the level in the sham-treated control rats (Fig. 2A) and NGF-treated rats (Fig. 2F). The level of NPY-LI fiber density after adrenomedullin administration (1000 ng/h)

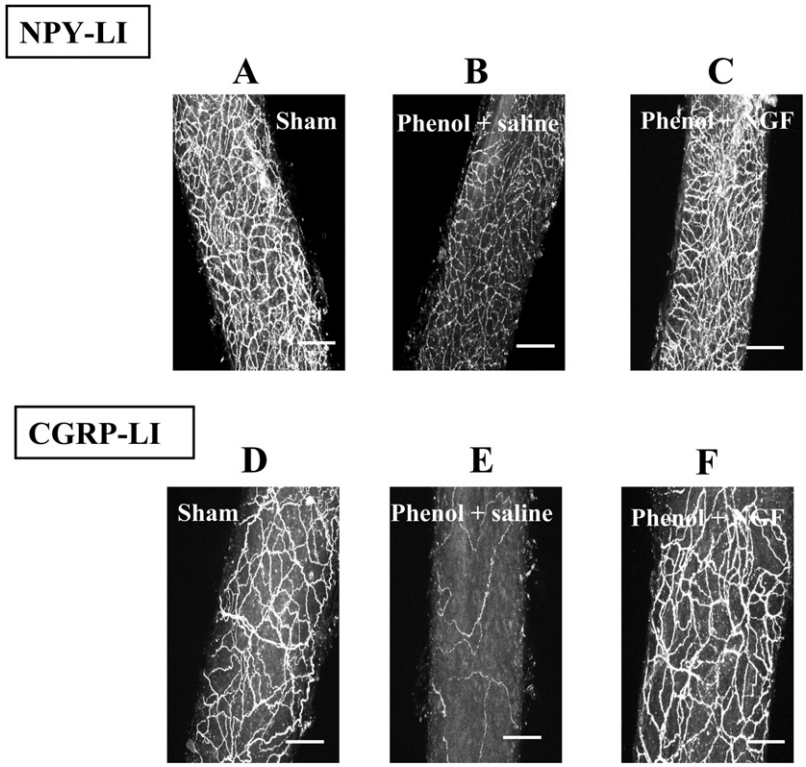

Fig. 1. Representative confocal laser micrographs showing changes in the density of NPY-LI nerve fibers and CGRP-LI nerve fibers in distal mesenteric arteries after topical phenol application on the superior mesenteric artery, and the effect of NGF. (A, D) Sham treatment control. (B, E) Density after phenol+saline treatment. (C, F) Density after phenol+NGF treatment. Scale bar=100 μm (lower right corner of each panel).

was significantly greater than that after phenol-saline treatment (Fig. 2D, F) and similar to those in the sham-treated control (Fig. 2A) and NGF-treated rats (Fig. 2E, F).

As shown in Fig. 3B, F, the density of CGRP-LI fibers markedly decreased by approximately 80% after topical phenol treatment of the superior mesenteric artery. The administration of adrenomedullin at 360 and 1000 ng/h caused significantly greater density of CGRP-LI nerve fibers than that in the phenol-saline-treated rats (Fig. 3C, D, F). However, the elevation of the CGRP-LI fiber density induced by adrenomedullin was smaller than that induced by NGF (Fig. 3E, F).

The relationships between the numbers of NPY-LI and CGRP-LI nerve fibers, which were counted visually, and the densities of NPY-LI and CGRP-LI nerves (%), which were quantified by computer-assisted image processing, were assessed in the mesenteric arteries of all groups. There were significant positive correlations between the density and the numbers of NPY-LI and CGRP-LI nerve fibers in the sham-treated control (NPY-LI, $P<0.01$, $r=0.762$; CGRP-LI, $P<0.01$, $r=0.585$), phenol-saline (NPY-LI, $P<0.01$, $r=0.740$; CGRP-LI, $P<0.01$, $r=0.822$), adrenomedullin 360 ng/h (CGRP-LI, $P<0.01$, $r=0.727$; NPY-LI, $P<0.01$, $r=0.629$), adrenomedullin 1000 ng/h (NPY-LI, $P<0.01$, $r=0.759$; CGRP-LI, $P<0.01$, $r=0.792$) and NGF (NPY-LI, $P=0.317$, $r=0.235$; CGRP-LI, $P<0.01$ $r=0.715$)-treated rats (data not shown).

Effect of treatment with adrenomedullin or NGF on adrenergic nerve-mediated vasoconstrictor responses to PNS and bolus injection of norepinephrine after topical phenol treatment

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 (Fig. 4). In the same preparation with resting tone, injection of norepinephrine caused a concentration-dependent vasoconstriction pattern similar to the PNS-induced response (Fig. 4). As shown in Figs. 4 and 5A, the vasoconstrictor responses to PNS at 8 and 12 Hz in preparations from phenol-saline-treated rats were significantly smaller than those in preparations from sham-treated control rats. The vasoconstrictor responses to norepinephrine injection in preparations from phenol-saline-treated rats tended to be greater than those in sham preparations, although there was no significant difference (Figs. 4 and 5B). In preparations from phenol-adrenomedullin (1000 ng/h)-treated rats (Figs. 4 and 5A) or phenol-NGF-treated rats (Figs. 5 A), the vasoconstrictor responses to PNS at 8 and 12 Hz were greater than those in preparations from phenol-saline-treated rats. There were significant differences in the PNS-induced response between the phenol-saline-treated rats and phenol-adrenomedullin- and phenol-NGF-treated rats (Fig. 5A). Furthermore, no significant difference was found between sham treatment groups and phenol-adrenomedullin

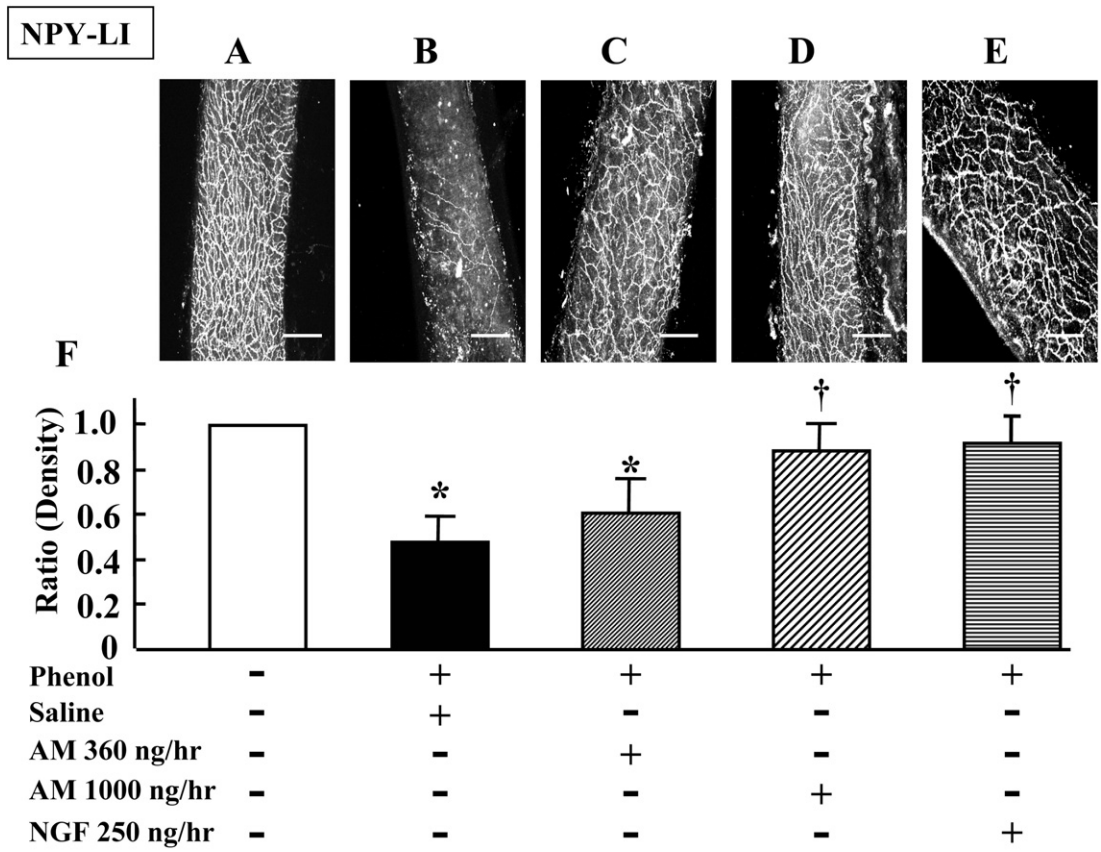

Fig. 2. Representative confocal-laser micrographs (A–E) and a bar graph (F) showing effects of adrenomedullin (AM) and NGF on changes in the density of NPY-LI nerve fibers in distal mesenteric arteries after topical phenol application on the superior mesenteric arteries. (A) Sham treatment control (n=10). (B) Phenol + saline treatment (n=12). (C) Phenol+AM 360 ng/h treatment (n=6). (D) Phenol+AM 1000 ng/h treatment (n=6). (E) Phenol+NGF 250 ng/h treatment (n=5). Each bar indicates mean±S.E.M. * $P<0.05$ vs. sham treatment control. † $P<0.05$ vs. saline-phenol treatment control.

or phenol-NGF treatment groups (Fig. 5A). However, in comparison with the preparations from the phenol-saline-treated rats, there was a significant reduction in the norepinephrine-induced vasoconstriction in the preparations from phenol-NGF-treated rats but not in those from phenol-adrenomedullin-treated rats (Figs. 4 and 5B). No significant difference in vasoconstrictor responses to norepinephrine injection was found between sham treatment groups and phenol-adrenomedullin or phenol-NGF treatment groups (Fig. 5B).

Effect of treatment with adrenomedullin or NGF on CGRPergic nerve-mediated vasodilator responses to PNS and bolus injection of CGRP after topical phenol treatment rats

To observe the vasodilator response, the preparation was induced to contract by continuous perfusion of methoxamine (7 μM; α_1-adrenergic receptor agonist) in the presence of guanethidine (5 μM; 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 (Fig. 4). The vasodilator response to PNS has been shown to be mediated by CGRP-containing vasodilator nerve, since the response is blocked by CGRP receptor antagonist [CGRP(8–37)] and CGRP depletor (capsaicin) (Han et al., 1990; Kawasaki et al.,1991). Bolus injections of CGRP also induced concentration-dependent vasodilation (Fig. 4), which has been shown to be mediated by postsynaptic CGRP receptors (Kawasaki et al., 1988).

Vasodilator responses to PNS at two and 4 Hz but not 1 Hz in preparations from phenol-saline-treated rats were significantly smaller than those in preparations from sham-treated control rats (Figs. 4 and 6A). As shown in Figs. 4 and 6A, the PNS (2 and 4 Hz)-induced vasodilator responses in preparations from phenol-adrenomedullin (1000 ng/h)-treated rats and phenol-NGF-treated rats tended to be larger than those of phenol-saline-treated rats but these effects did not reach the level of statistical significance ($P<0.1$). However, there was no difference between neurally evoked vasodilator response of preparations from the sham treatment control group and those from phenol-adrenomedullin and phenol-NGF treatment groups (Fig. 6 A).

As shown in Figs. 4 and 6B, vasodilator responses to CGRP injection did not change after phenol-saline treatment. In preparations from phenol-adrenomedullin-treated

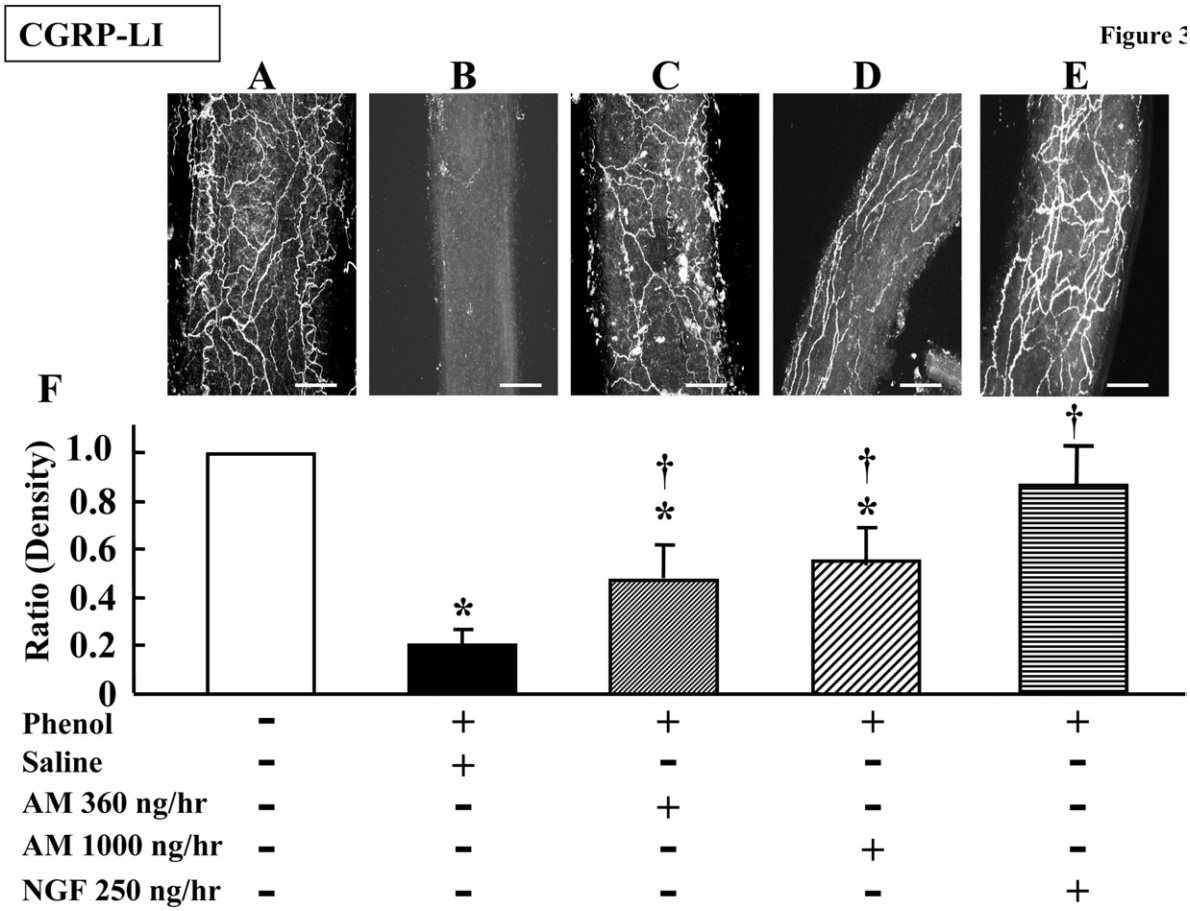

Fig. 3. Representative confocal-laser micrographs (A–E) and bar graph (F) showing effects of adrenomedullin (AM) and NGF treatment on changes in the density of CGRP-LI nerve fibers in the distal mesenteric artery after topical phenol treatment of the superior mesenteric artery. (A) Sham treatment control (n=10). (B) Phenol+saline treatment (n=12). (C) Phenol+AM 360 ng/h treatment (n=6). (D) Phenol+AM 1000 ng/h treatment (n=6). (E) Phenol+NGF 250 ng/h treatment (n=5). Each bar indicates mean±S.E.M. * $P<0.05$ vs. sham treatment control. † $P<0.05$ vs. saline-phenol treatment control.

rats (Figs. 4 and 6B), the vasodilator response to CGRP injection was similar to that in preparations from sham-treated control and phenol-saline-treated rats. However, the vasodilator response to CGRP injection in preparations from phenol-NGF-treated rats was significantly smaller than those in preparations from sham-treated control rats and phenol-saline-treated rats (Figs. 4 and 6B).

DISCUSSION

The present study is the first report to demonstrate that adrenomedullin, a potent vasodilator peptide, facilitates the reinnervation of perivascular NPY-LI and CGRP-LI nerves in the rat mesenteric artery which was injured by topical application of phenol. Our recent report provided evidence that topical treatment of the rat superior mesenteric artery with phenol induces a marked reduction of perivascular NPY- and CGRP-containing nerve innervation in the distal small artery. Additionally, NGF treatment for 7 days immediately after phenol application restored the innervation of perivascular nerves to the control level. These findings were confirmed by the present results that topical phenol application reduced the density of perivascular NPY-LI and CGRP-LI nerves in the mesenteric artery, and that this reduction was prevented by NGF treatment. Furthermore, the present findings showed that adrenomedullin cancelled the phenol-induced reduction of the innervation of both NPY-LI and CGRP-LI nerves, suggesting that adrenomedullin, like NGF, has the ability to enhance reinnervation of the perivascular nerves. It is assumed that the reinnervation process induced by adrenomedullin and NGF may be due to sprouting of the remaining axons, since the effects of both adrenomedullin and NGF were seen only 7 days following the proximal lesion.

The topical phenol treatment resulted in marked reduction of not only the innervation of NPY-LI-containing nerves but also the PNS-induced vasoconstrictor response, which is mediated by perivascular adrenergic nerves. 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 norepinephrine (Lundberg, 1996).

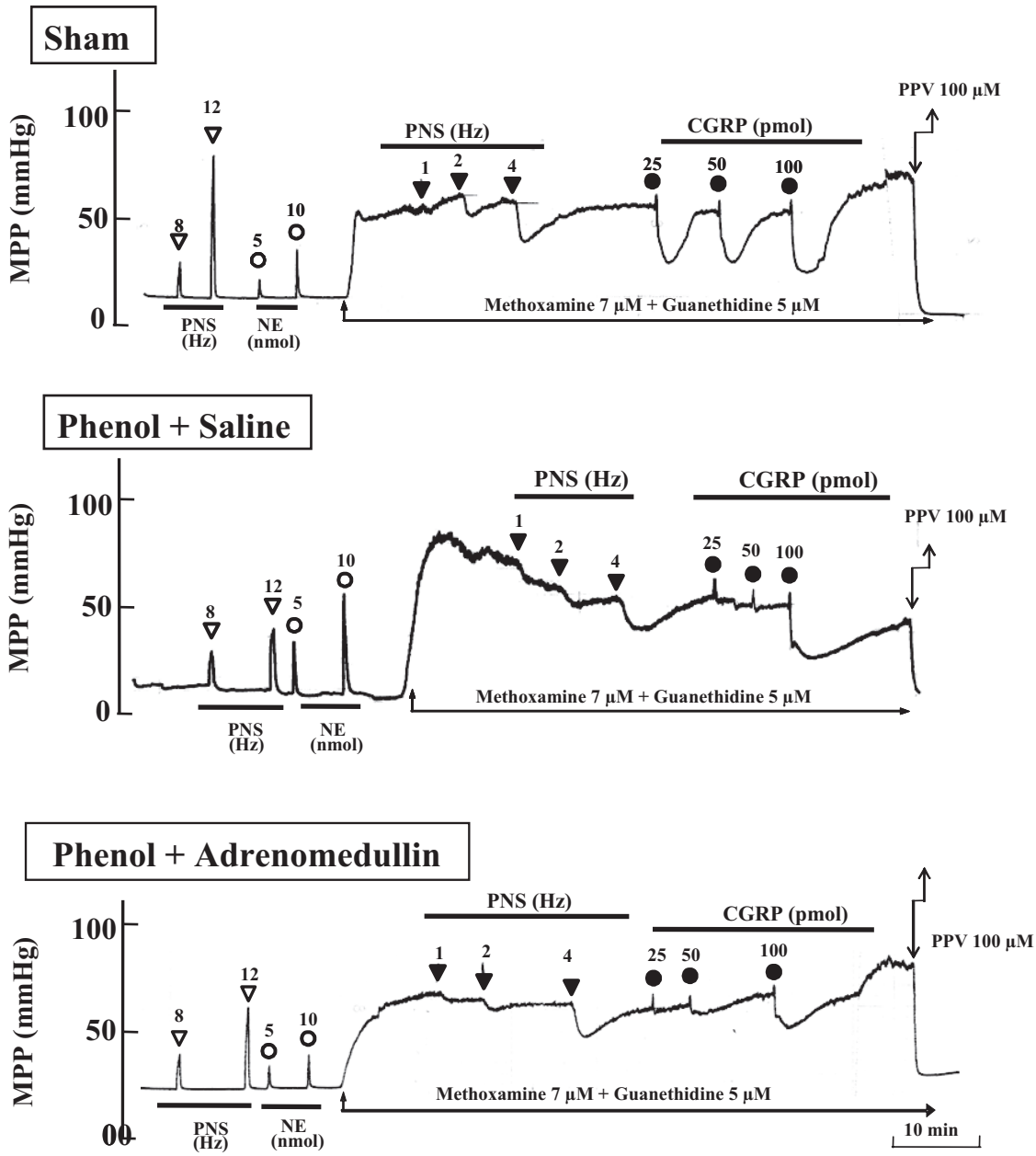

Fig. 4. Typical records showing the effect of adrenomedullin treatment on changes in vasoconstrictor and vasodilator responses to PNS and to bolus infusion of norepinephrine (NE, 5 and 10 nmol) and rat CGRP (25, 50 and 100 pmol) after topical phenol application in perfused mesenteric vascular beds with active tone produced by 7 μM methoxamine in the presence of 5 μM guanethidine. Sham, the preparation isolated from sham-treated control rats; phenol+saline, the preparation isolated from phenol-saline-treated rats; phenol+adrenomedullin, the preparation isolated from phenol-adrenomedullin-treated rats. Inverted open triangles and open circles show PNS at 8 and 12 Hz and bolus injections of NE at 5 and 10 nmol, respectively. Inverted closed triangles and closed circles indicate PNS at 1, 2 and 4 Hz and bolus injections of CGRP at 25, 50 and 100 nmol, respectively.

Therefore, it is likely that phenol treatment damaged the sympathetic postganglionic NPY-LI nerve trunk innervating the small mesenteric artery, and led to a significant decrease in the neurogenic vasoconstriction induced by PNS. Treatment of phenol-treated rats with adrenomedullin significantly restored the PNS-induced vasoconstriction to the level in the sham-treated control and NGF-treated rats. This result is in good accordance with the restoration of the innervation of NPY-LI nerve fibers, suggesting that the NPY-LI nerves that reinnervated the artery after treatment with adrenomedullin or NGF functioned as perivascular nerves.

The rat mesenteric artery is densely innervated by CGRP-LI nerves, in which PNS induces vasodilation (Kawasaki et al., 1988). The present finding that phenol treatment decreased CGRP nerve-mediated vasodilation in response to PNS suggests that ablation and functional loss of perivascular CGRP-LI nerves occurred following topical

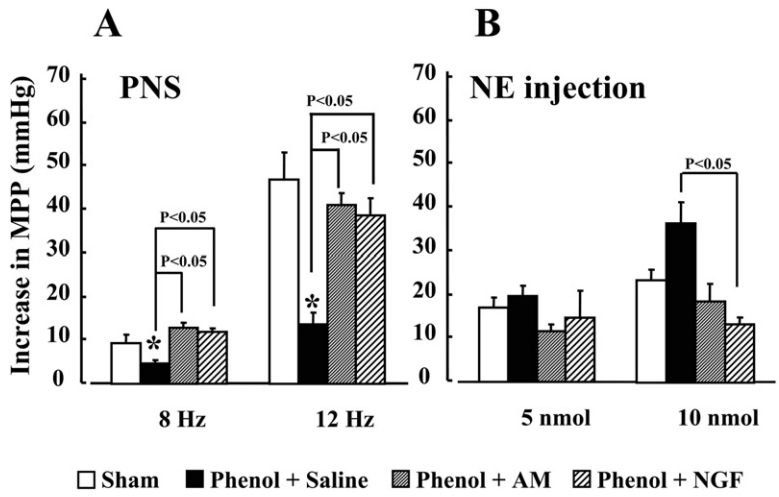

Fig. 5. Effects of adrenomedullin (AM) and NGF treatment on changes in adrenergic nerve-mediated vasoconstrictor responses to PNS (A) and to bolus infusion of norepinephrine (NE) (B) in perfused mesenteric vascular beds after topical phenol treatment. Sham (n=10), the preparation isolated from sham-treated control rats; phenol+saline (n=12), the preparation isolated from phenol-saline-treated rats; phenol+AM (n=6), the preparation isolated from phenol-adrenomedullin (1000 ng/h)-treated rats; phenol+NGF (n=5), the preparation isolated from phenol-NGF (250 ng/h)-treated rats. Each bar indicates mean±S.E.M. * P<0.05 vs. sham treatment control.

phenol application. Adrenomedullin tended to increase the PNS-induced vasodilation that had been decreased after phenol application. Adrenomedullin increased the level of CGRP-LI nerve fibers to the control level, indicating that the peptide also has neurotrophic activity for perivascular CGRP-containing nerves. Additionally, NGF treatment increased the PNS-induced vasodilation to return it back to that of the sham treatment group. However, the reverse effect of NGF on the neurally evoked vasodilation was smaller than that of adrenomedullin. NGF treatment resulted in a significantly decreased vasodilator response to exogenously applied CGRP compared with the responses of the sham-treated control and phenol-treated preparations. Therefore, the weaker effectiveness of NGF treatment on the PNS-induced vasodilation may be in part associated with decreased vasodilation in response to exogenous CGRP, which was induced by activation of postsynaptic CGRP receptors. NGF induces up-regulation of CGRP synthesis in the dorsal root ganglia neurons (Lindsay and Harmar, 1989; Supowit et al., 2001). Furthermore, endoneurial injection of NGF increased the CGRP expression in the dorsal root ganglia (Schuligoi and Amann, 1998; Ruiz and Banos, 2005). In the present study, NGF markedly increased the density of CGRP-LI

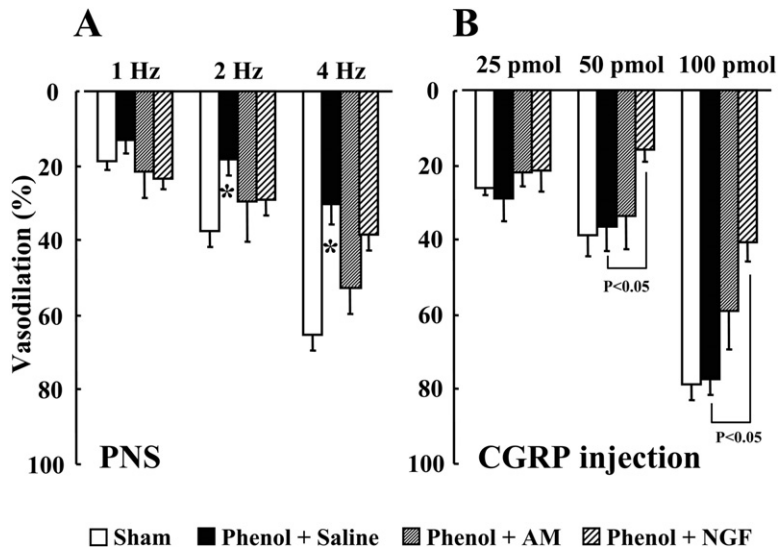

Fig. 6. Effects of adrenomedullin (AM) and NGF treatment on changes in CGRP nerve-mediated vasodilator responses to PNS (A) and to bolus infusion of CGRP (B) in perfused mesenteric vascular beds after topical phenol treatment. Sham (n=10), the preparation isolated from sham-treated control rats; phenol+saline (n=12), the preparation isolated from phenol-saline-treated rats; phenol+AM (n=6), the preparation isolated from phenol-adrenomedullin (1000 ng/h)-treated rats; phenol+NGF (n=5), the preparation isolated from phenol-NGF (250 ng/h)-treated rats. Each bar indicates mean ± S.E.M. * P<0.05 vs. sham treatment control.

nerve fibers in the phenol-treated rats. Therefore, it is presumed that NGF may down-regulate CGRP receptor expression in the mesenteric artery.

Khan et al. (1997) reported that chronic administration of adrenomedullin at a rate of 1000 ng/h significantly lowered the blood pressure in two-kidney, one-clip hypertensive rats. Our previous reports demonstrated age-related decreases in CGRP nerve-mediated vasodilation (Kawasaki et al., 1990), neurogenic CGRP release (Kawasaki et al., 1990), the distribution of CGRP-LI nerve fibers in the mesenteric artery (Hobara et al., 2005) and CGRP mRNA levels in the dorsal root ganglia (Kawasaki et al., 2001) in spontaneously hypertensive rats (SHRs). We suggested that remodeling of CGRP-containing vasodilator nerve fibers contributes to the development and maintenance of hypertension in SHRs. Furthermore, SHRs and hypertensive patients have been shown to have elevated plasma levels of adrenomedullin (Kohno et al., 1996). The main source of plasma adrenomedullin has been shown to be vascular endothelium and smooth muscle cells (Sugo et al., 1994). Therefore, the present finding that adrenomedullin facilitates the distribution of perivascular CGRP nerves and restores the CGRP nerve-mediated vascular responses implies that in hypertension, adrenomedullin may act to prevent and/or restore the perivascular nerve remodeling as a neurotrophic factor.

CONCLUSION

The present results suggest that adrenomedullin facilitates the reinnervation of perivascular NPY-LI and CGRP-LI nerve fibers injured by topical phenol application. The results also suggest that adrenomedullin, like NGF, has the ability to regenerate and/or reinnervate perivascular nerves as a neurotophic factor and that adrenomedullin may play an important role in preventing the remodeling of perivascular nerves.

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REFERENCES

Asada Y, Hara S, Marutsuka K, Kitamura K, Tsuji T, Sakata J, Sato Y, Kisanuki A, Eto T, Sumiyoshi A (1999) Novel distribution of adrenomedullin-immunoreactive cells in human tissues. Histochem Cell Biol 112:185–191.

Chena L, Kisb B, Busijab DW, Yamashitac H, Ueta Y (2005) Adrenomedullin protects rat cerebral endothelial cells from oxidant damage in vitro. Regul Pept 130:27–34.

Dogan A, Suzuki Y, Koketsu N, Osuka K, Saito K, Takayasu M (1997) Intravenous infusion of adrenomedullin and increase in regional cerebral blood flow and prevention of ischemic brain injury after middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab 17:19–25.

Dumoulin FL, Raivich G, Haas CA, Lazar P, Reddington M, Streit WJ, Kreutzberg GW (1992) Calcitonin gene-related peptide and peripheral nerve regeneration. Ann N Y Acad Sci 657:351–360.

Han SP, Naes L, Westfall TC (1990) Inhibition of periarterial nerve stimulation-induced vasodilation of the mesenteric arterial bed by CGRP (8–37) and CGRP receptor desensitization. Biochem Biophys Res Commun 168:786–791.

Hobara N, Gessei-Tsutsumi N, Goda M, Takayama F, Akiyama S, Kurosaki Y, Kawasaki H (2005) Long-term inhibition of angiotensin prevents reduction of periarterial innervation of calcitonin gene-related peptide (CGRP) containing nerves in spontaneously hypertensive rats. Hypertens Res 28:465–474.

Hobara N, Goda M, Takayama F, Kawasaki H (2006) Innervation and functional changes in mesenteric perivascular calcitonin gene-related peptide (CGRP)- and neuropeptides Y (NPY)-containing nerves following topical phenol treatment. Neuroscience 141: 1087–1099.

Hobara N, Nakamura A, Ohtsuka A, Narasaki M, Shibata K, Gomoita Y, Kawasaki H (2004) Distribution of adrenomedullin-containing perivascular nerves in the rat mesenteric artery. Peptides 25: 589–599.

Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, Eto T (1994) Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. FEBS Lett 338:6–10.

Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, Matsuo H, Eto T (1994) Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. Biochem Biophys Res Commun 200:642–646.

Israel A, Diaz E (2000) Diuretic and natriuretic action of adrenomedullin administered intracerebroventricularly in conscious rats. Regul Pept 89:13–18.

Jougasaki M, Wei CM, Aarhus LL, Heublein DM, Sandberg SM, Burnett JC Jr (1995) Renal localization and actions of adrenomedullin: a natriuretic peptide. Am J Physiol 268:F657–F663.

Kawasaki H, Nuki C, Saito A (1991) NPY modulates neurotransmission of CGRP-containing vasodilator nerves in rat mesenteric arteries. Am J Physiol 261:H683–H690.

Kawasaki H, Nuki Y, Yamaga N, Kurosaki Y, Taguchi T (2001) decreased depressor response mediated by calcitonin gene-related peptide (CGRP)-containing vasodilator nerves to spinal cord stimulation and levels of CGRP mRNA of the dorsal root ganglia in spontaneously hypertensive rats. Hypertens Res 23:693–699.

Kawasaki H, Saito A, Takasaki K (1990) Age-related decrease of calcitonin gene-related peptide-containing vasodilator innervation in the mesenteric resistance vessel of the spontaneously hypertensive rat. Circ Res 67:733–743.

Kawasaki H, Takasaki K, Saito A, Goto K (1988) Calcitonin gene-related peptide acts as a neurotransmitter in mesenteric resistance vessels of the rat. Nature 335:164–167.

Kawasaki H, Takenaga M, Araki H, Futagami K, Gomita Y (1998) Angiotensin inhibits neurotransmission of calcitonin gene-related peptide-containing vasodilator nerves in mesenteric artery of spontaneously hypertensive rats. J Pharmacol Exp 284:508–515.

Khan AI, Kato J, Ishiyama Y, Kitamura K, Kangawa K, Eto T (1997) Effect of chronically infused adrenomedullin in two-kidney, one-clip hypertensive rats. Eur J Pharmacol 333:187–190.

Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 192:553–560.

Kohno M, Hanehira T, Kano H, Horio T, Yokokawa K, Ikeda M, Minami M, Yasunari K (1996) Plasma adrenomedullin concentrations in essential hypertension. Hypertension 27:102–107.

Lee Y, Takami K, Kawai Y, Girgis S, Hillyard CJ, MacIntyre I, Emspon PC, Tohyama M (1985) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. Neuroscience 15:1227–1237.

Lindsay RM, Harmar AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337:362–364.

Lundberg JM (1996) Pharmacology of co-transmission in the autonomic nervous system: Integrative aspects on amines neuropeptides adenosine 5′-triphosphate amino acids and nitric oxide. Pharmacol Rev 48:113–178.

Marti E, Gibson SJ, Polak MN (1987) Ontogeny of peptide and amine-containing neurons in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia and rat skin. J Comp Neurol 266:332–359.

Miyashita K, Itoh H, Arai H, Suganami T, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, Oyamada N, Sawada N, Taura D, Tsujimoto H, Chao TH, Tamura N, Mukoyama M, Nakao K (2006) The neuroprotective and vasculo-neuro-regenerative roles of adrenomedullin in ischemic brain and its therapeutic potential. Endocrinology 147:1642–1653.

Murphy TC, Samson WK (1995) The novel vasoactive hormone, adrenomedullin, inhibits water drinking in the rat. Endocrinology 136:2459–2463.

Nagaya N, Kangawa K (2004) Adrenomedullin in the treatment of pulmonary hypertension. Peptides 25:2013–2018.

Nuki C, Kawasaki H, Kitamura K, Takenaga M, Kangawa K, Eto T, Wada A (1993) Vasodilator effect of adrenomedullin and calcitonin gene-related peptide receptors in rat mesenteric vascular beds. Biochem Biophys Res Commun 196:245–251.

Piehl F, Hammarberg H, Tabar G, Hökfelt T, Cullheim S (1998) Changes in the mRNA expression pattern, with special reference to calcitonin gene-related peptide, after axonal injuries in rat motoneurons depends on age and type of injury. Exp Brain Res 119:191–204.

Ruiz G, Banos JE (2005) The effect of endoneurial nerve growth factor on calcitonin gene-related peptide expression in primary sensory neurons. Brain Res 1042:44–52.

Samson WK, Murphy TC (1997) Adrenomedullin inhibits salt appetite. Endocrinology 138:613–616.

Schuligoi R, Amann R (1998) Differential effects of treatment with nerve growth factor on thermal nociception and on calcitonin gene-related peptide content of primary afferent neurons in the rat. Neuroscience Lett 252:147–149.

Serrano J, Uttenthal LO, Martinez A, Fernandez AP, Martinez de Velasco J, Alonso D, Bentura ML, Santacana M, Gallardo JR, Martinez-Murillo R, Cuttitta F, Rodrigo J (2000) Distribution of adrenomedullin-like immunoreactivity in the rat central nervous system by light and electron microscopy. Brain Res 853:245–268.

Sugo S, Minamino N, Kangawa K, Miyamoto K, Kitamura K, Sakata J, Eto T, Matsuo H (1994) Endothelial cells actively synthesize and secrete adrenomedullin. Biochem Biophys Res Commun 201: 1160–1166.

Supowit SC, Zhao H, DiPette DJ (2001) Nerve growth factor enhances calcitonin gene-related peptide expression in the spontaneously hypertensive rat. Hypertension 37:728–732.

Wang Y, Bukoski RD (1999) Use of acute phenolic denervation to show the neuronal dependence of Ca^{2+}-induced relaxation of isolated arteries. Life Sci 64:887–894.

Watanabe K, Takayasu M, Noda A, Hara M, Takagi T, Suzuki Y (2001) Adrenomedullin reduces ischemic brain injury after transient middle cerebral artery occlusion in rats. Acta Neurochir 143: 1157–1161.

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