Human Anatomy & Physiology

Neuroscience

Okayama University *Year* 2008

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Angiotensin II type 2 receptors facilitate reinnervation of phenol-lesioned vascular calcitonin gene-related peptide (CGRP)-containing nerves in rat mesenteric arteries

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Short title: AT2 receptor and reinnervation

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ABSTRACT

The present study was designed to investigate involvement of angiotensin (Ang) II type 2 receptors (AT2 receptors) in restoration of perivascular nerve innervation injured by topical phenol treatment. Male Wistar rats underwent *in vivo* topical application of 10% phenol around the superior mesenteric artery. After phenol treatment, animals were subjected to immunohistochemistry of the third branch of small arteries, Western blot analysis of AT2 receptor protein expression in dorsal root ganglia (DRG) and studies of mesenteric neurogenic vasoresponsiveness. Ang II (750 ng/kg/day), nerve growth factor (NGF; 20 μg/kg/day) and PD123,319 (AT2 receptor antagonist; 10 mg/kg/day) were intraperitoneally administered for 7 days using osmotic mini-pumps immediately after topical phenol treatment. Losartan (AT1 receptor antagonist) was administered in drinking water (0.025%). Phenol treatment markedly reduced densities of both calcitonin gene-related peptide (CGRP)-like immunoreactivity (LI)- and neuropeptide Y (NPY)-LI-containing fibers. NGF restored densities of both nerve fibers to the Sham control level. Coadministration of Ang II and losartan significantly increased the density of CGRP-LI-fibers but not NPY-LI-fibers compared with saline control. The increase of the density of CGRP-LI-fibers by coadministration of Ang II and losartan was suppressed by adding PD123,319. Coadministration of Ang II and losartan ameliorated reduction of CGRP nerve-mediated vasodilation of perfused mesenteric arteries caused by phenol treatment. The AT2 receptor protein expression detected in DRG was markedly increased by NGF. These results suggest that selective stimulation of AT2 receptors by Ang II facilitates reinnervation of mesenteric perivascular CGRP-containing nerves injured by topical phenol application in the rat.

Key Words: Angiotensin II type 2 receptors; Phenol-induced perivascular nerve injury; Calcitonin gene-related peptide-containing nerves; Neuropeptide Y-containing nerves; Neurotrophic; Rat mesenteric artery

List of abbreviations

Ang II: angiotensin II

AT1: angiotensin II type 1

AT2: angiotensin II type 2

CGRP: calcitonin gene-related peptide

DRG: dorsal root ganglia

NE: norepinephrine

NGF: nerve growth factor

NPY: neuropeptide Y

PNS: periarterial nerve stimulation

LI: like immunoreactivity

PNS: perivascular nerve stimulation

PBS: phosphate-buffered saline

It is generally accepted that angiotensin (Ang) II acts on two subtype receptors, which are Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors. AT1 receptors mediate the main physiological actions of Ang II, including vasoconstriction, facilitation of sympathetic nerve function and enhancement of cell proliferation, while AT2 receptors have opposite physiological effects from those of AT1receptors, i.e., vasodilation, cell apoptosis and inhibition of cell proliferation (Nakajima et al., 1995; Stoll et al., 1995; Stroth et al., 1998). Growth-promoting effects of Ang II have been shown to be mediated primary via AT1 receptors (Touyz and Schiffrin, 1997). However, AT2 receptors are involved in neuronal differentiation of PC12W cells (Meffert et al., 1996; Gallinat et al., 1997). Furthermore, there is a growing body of evidence that stimulation of AT2 receptors promotes cell differentiation and regeneration in neuronal tissues (Gasparo et al., 2000; Reinecke et al., 2003). AT2 receptors are drastically up regulated under conditions such as tissue injury (Viswanathan and Saavedra, 1992) or sciatic nerve axotomy (Gallinat et al., 1998). Several studies have suggested that Ang II, via the AT2 receptors, acts as a neurotrophic factor for peripheral neurons (Lucius et al., 1998; Reinecke et al., 2003).

Perivascular nerves play an important role in the maintenance and regulation of vascular tone. The mesenteric artery, which is part of a large vascular bed, has dense innervation of perivascular nerves such as sympathetic adrenergic vasoconstrictor nerves and non-adrenergic non-cholinergic calcitonin gene-related peptide (CGRP) containing nerves (CGRPergic nerves), which act as vasodilator nerves (Kawasaki et al., 1988, 1990a). Previous studies demonstrated that the innervation and function of CGRPergic nerves in mesenteric resistance arteries of spontaneously hypertensive rats (SHR) decrease with ageing to cause CGRPergic nerve remodeling (Kawasaki et al., 1990b; Kawasaki and Takasaki, 1992;

Kawasaki et al., 2001). Furthermore, long-term treatment with AT1 receptor antagonist prevents the CGRPergic nerve remodeling in SHR (Kawasaki et al., 2003; Hobara et al., 2005), implying that Ang II is an active substance that induces perivascular nerve remodeling via AT1 receptors. Thus, those studies led to the hypothesis that the restoration of CGRPergic nerve innervation after blockade of AT1 receptors in SHR might be due to stimulation of AT2 receptors, which exert a neurotrophic effect on the reinnervation.

Recently, we demonstrated that innervation of CGRP- and neuropeptide Y (NPY)-containing adrenergic 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 stimulation of AT2 receptors has the ability to facilitate reinnervation of mesenteric perivascular nerves after *in vivo* denervation induced by topical treatment with phenol (Hobara et al., 2006). To stimulate AT2 receptors, Ang II was administered in the presence of an AT1 receptor antagonist (losartan) after topical phenol treatment and innervation and functional changes in perivascular CGRP- or NPY-containing nerves in rat mesenteric resistance arteries 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/12-h 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.

Animal treatments and experimental protocols

After anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), an abdominal midline incision was made in the animal, 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. Sham-operated rats underwent the same surgical procedures, except for swabbing with vehicle (saline or 90% alcohol without including phenol) instead of phenol solution. After the swabbing, a mini-osmotic pump (model 2001, Alzet, Alza, Palp Alto, CA, USA) was implanted in the abdominal area to administer human recombinant Ang II (Peptide Institute, Osaka, Japan) at a rate of 60 μg/kg/day, PD123,319 (Sigma Aldrich Japan, Tokyo, Japan) at a rate of 10 mg/kg/day or NGF (Sigma Aldrich Japan) at a rate of 20 μg/kg/day for a period of 7 days. Ang II, PD123,319 and NGF were dissolved in sterile saline and injected into Alzet mini-osmotic pumps. Losartan (Merck & Co., Inc., Rahway, USA) was dissolved in drinking water at a concentration of 0.025 %. Control animals were implanted with mini-osmotic pumps containing sterile saline alone. Ang II at a dose of 60 μg/kg/day was

administered, which increased the systolic blood pressure. The doses of losartan and NGF and PD123,319 were used according to our previous reports (Hobara et al., 2005 and 2006) and the report described by Jones et al. (2004), respectively. After the swabbing and implanting, an antibiotic (penicillin G; Sigma Aldrich 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, except for swabbing with vehicle (saline or 90% alcohol without 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 3 consecutive days. After phenol treatment and sham operation, the animals were killed by deep anesthesia on Day 7 for use in the experiments described below.

The animals were randomly divided into six groups: (1) normal control group (Sham); (2) phenol-saline group (Ph + Saline), animals receiving saline after phenol treatment; (3) phenol-Ang II group (Ph + Ang II), animals receiving Ang II after phenol treatment; (4) phenol-Ang II-losartan group (Ph + Ang II + Los), animals receiving both Ang II and losartan after phenol treatment; (5) phenol-Ang II-losartan-PD123,319 group (Ph + Ang II + Los + PD), animals concomitantly receiving Ang II, losartan and PD123,319 after phenol treatment; (6) phenol-NGF group (Ph +NGF), animals receiving NGF after phenol treatment.

Systolic blood pressure measurement

The systolic blood pressure 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 topically with phenol or vehicle were anesthetized with a large dose of sodium pentobarbital (50 mg/kg, intraperitoneally). The superior mesenteric artery was cannulated with polyethylene tubing and Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer) was infused, 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 rabbit polyclonal anti-CGRP (Biogenesis Ltd., Oxford, UK) antiserum at a dilution of 1:300 or rabbit polyclonal anti-NPY (Phoenix Pharmaceuticals INC., Belmont, CA, U.S.A.) antiserum at a dilution of 1:300 for 72 h at 4ºC. After the incubation, the artery was washed in PBS and the sites of antigen-antibody reaction were detected 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 artery was thoroughly washed in PBS, mounted on a slide, cover-slipped with glycerol/PBS (2: 1 v/v) and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss, Tokyo, Japan) in Okayama University Medical School Central Research Laboratory.

Immunohistochemical analysis

The immunostaining density of CGRP-like immunoreactive (CGRP-LI)

and NPY-like immunoreactive (NPY-LI) nerve fibers was analyzed using the method described by Hobara et al. (2005 and 2006). Since the fluorescence intensity differed depending on the day of the experiment, the mesenteric arteries from rats treated with each drug after topical phenol treatment on Day 7 and vehicle-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 vehicle-treated rats at Day 7 were used as a control for the intensity in each experiment. For quantitative evaluation of CGRP-LI and NPY-LI, confocal projection images of CGRP and NPY immunostaining, which consisted of 8-10 overlapping images (0.1 μm scanning) patched together, were magnified 20x 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 CGRP-LI and NPY-LI areas (which were stained green). Extraction of the signal was carried out using specific protocols based on the hue, lightness, and saturation color parameters. A measured field of 100 μm x 100 μm (10000 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 CGRP-LI- and NPY-LI-positive area. 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 3 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, 1990a). 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 on 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), and then was contracted with α1-adrenoceptor agonist methoxamine (7 μM) in the presence of an adrenergic neuron blocker, guanethidine (5 μM), 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). 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 injected directly 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, preparations were perfused with 100 μM papaverine to cause complete relaxation. Vasodilator activity is expressed as the percentage of the perfusion pressure at the maximum relaxation induced by papaverine. Vasoconstrictor activity is expressed as the percentage of the perfusion pressure.

Western blot analysis of AT2 receptor protein

The DRG was homogenized with scissors in 300 μl of Tris-buffered saline (20 mM Tris HCl (pH 7.4), 1 mM EDTA) containing protease inhibitor cocktail (Biovision Research Products, Mountain View, CA,

USA). The homogenate was centrifuged at 600 g for 10 min at 4°C. The supernatant was further centrifuged at 100,000 g for 1 h at 4°C. The resulting pellet was then washed and resuspended with the same Tris-buffered saline with 0.1 % Triton-X. The solution was centrifuged at 100,000 g for 1 h at 4 °C. The concentration of protein in homogenate was determined using Bio-Rad protein assay solution (Bio-Rad Laboratories, Inc., Osaka, Japan) with bovine serum albumin as a standard. For Western blotting, membrane proteins were electrophoresed on a standard 12 % sodium deodecyl sulfate (SDS)-polyacrylamide gel (Bio-Rad Laboratories) in Tris-glycine electrophoresis buffer (25 mM Tris, 192 mM glycine (pH 8.3), and 0.1 % SDS). Proteins were transferred onto a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK) in 192 mM glycine, 25 mM Tris (pH 8.3), and 30 % methanol at 100 v for 1.5 h. The membrane was blocked in a blocking buffer (PBS containing 10% goat serum and 0.01M EDTA) at room temperature for 1 h. The membrane was then probed overnight at 4 °C with polyclonal antibody against AT2 receptor (Alpha Diagnostic International, San Antonio, TX, USA) (1:1000) or polyclonal antibody against β-actin (Cell Signaling Technology, Inc, Danvers, MA, USA) (1:2000) in the blocking buffer. After the membrane was washed five times in PBS it was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:1000; R&D Systems, Inc., Minneapolis, MN, USA) in the blocking buffer for 1 h at room temperature. Bound antibodies were detected using a chemiluminescent substrate kit (Amersham Biogensesis) and the content of β-actin was used as a control to ensure that the same amount of protein was loaded in each lane.

Reagents

The following drugs were used: losartan (Merck & Co., Inc., Rahway, NJ, USA), NGF (Sigma Aldrich Japan), PD123,319 (Sigma Aldrich Japan) and penicillin G (Sigma Aldrich Japan), Ang II (Peptide Institute, INC., Osaka, Japan).

Statistical Analysis

All data are expressed as mean ± S.E.M. Analysis of variance (ANOVA) followed by the 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.

RESULTS

Changes in systolic blood pressure (SBP) after phenol treatment

Fig. 1 shows the tail-cuff systolic blood pressure in the six groups for 7 days after topical phenol or vehicle (Sham) treatment. The Ang II group (Ph + Ang II) showed significantly increased blood pressure from 4 days after Ang II administration compared with the saline control group (Ph + saline). Losartan administration (Ang II + Los and Ang II + Los + PD) completely inhibited the increase of blood pressure induced by Ang II.

Changes in innervation of CGRP-LI nerve fibers in mesenteric arteries following topical phenol treatment with or without administration of each drug

Fig. 2 and Fig. 3 show typical images of innervation of CGRP-LI nerves and changes in the density of CGRP-LI nerve fibers in the mesenteric artery after topical treatment with phenol and 7-day treatment with each

drug, respectively. As shown in Fig. 2A, the distal small mesenteric artery from the Sham group, which was treated topically with vehicle (saline or 90% alcohol solution without phenol) on the superior mesenteric artery, had dense innervation of CGRP-LI nerves. Topical application of phenol on the superior mesenteric artery, as shown in Fig. 2B, caused a marked reduction of innervation of CGRP-LI nerve fibers in the distal small mesenteric artery. The density of CGRP-LI nerve fibers in the phenol-saline-treated group was significantly decreased to approximately 20% of that in the Sham group (Fig. 3).

Administration of Ang II caused a 20% increase in the density of CGRP-LI nerve fibers, but no significant difference was found between the Ang II group and phenol-saline group, as shown in Fig. 2C and Fig. 3. Combined administration of Ang II and an AT1 receptor antagonist, losartan, (Ph + Ang II + Los) after topical phenol treatment significantly elevated the density of CGRP-LI nerve fibers compared with that in the phenol-saline group (Ph + saline) (Fig. 2D and Fig. 3). However, when PD123,319, an AT2 receptor antagonist, was combined with Ang II and losartan (Ph + Ang II + Los + PD), the density of CGRP-LI nerve fibers decreased to a level similar to that in the phenol-saline group (Fig. 2E and Fig. 3).

The relationships between the numbers of CGRP-LI nerve fibers, which were counted visually, and the densities of 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 CGRP-LI nerve fibers in the Sham ($p<0.01$, $r = 0.801$), phenol + saline ($p<0.01$, $r = 0.943$), phenol + Ang II ($p<0.01$ $r = 0.833$), phenol + Ang II + losartan ($p<0.01$, $r = 0.890$), and phenol + Ang II + losartan + PD123,319 ($p<0.01$, $r = 0.859$) groups

(data not shown).

Changes in innervation of NPY-LI nerve fibers in mesenteric arteries following topical phenol treatment with or without administration of each drug

Fig. 4 and Fig. 5 show typical images of innervation of NPY-LI nerves and changes in the density of NPY-LI nerve fibers in the mesenteric artery after topical treatment with phenol and 7-day treatment with each drug, respectively. As shown in Fig. 4A, the distal small mesenteric artery from the Sham group had denser innervation of NPY-LI nerves than CGRP-LI nerve fibers. Topical phenol application on the superior mesenteric artery reduced innervation of NPY-LI nerve fibers in the distal small mesenteric artery (Fig. 4B). The density of NPY-LI nerve fibers in the phenol-saline treated group was significantly decreased to approximately 50% of that in the sham group (Fig. 5).

Administration of Ang II after phenol treatment caused approximately a 15% increase in the density of NPY-LI nerve fibers, but no significant difference was found between this group and the phenol-saline group, as shown in Fig. 4C and Fig. 5. Combined administration of Ang II and losartan (Ph + Ang II + Los) or Ang II, losartan and PD123,319 (Ph + Ang II + Los + PD) after topical phenol treatment did not significantly affect the density of NPY-LI nerve fibers compared with that in the phenol-saline group (Ph + saline) (Fig. 4D, Fig. 4E and Fig. 5).

The relationships between the numbers of NPY-LI nerve fibers, which were counted visually, and the densities of NPY-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 nerve fibers in

the Sham ($p<0.01$, $r = 0.728$), phenol + saline ($p<0.01$, $r = 0.827$), phenol + Ang II ($p<0.01$ $r = 0.595$), phenol + Ang II + losartan ($p<0.01$, $r = 0.849$) and phenol + Ang II + losartan + PD123,319 ($p<0.01$, $r = 0.889$) groups (data not shown).

Effect of NGF on changes in innervation of CGRP-LI and NPY-LI nerve fibers in mesenteric arteries following topical phenol treatment

The administration of NGF after topical phenol administration markedly elevated the density of both CGRP-LI (Fig. 2F and Fig. 3) and NPY-LI nerve fibers (Fig. 4F and Fig. 5) to levels similar to those in the Sham group. There were significant differences in the densities of CGRP-LI nerve fibers between the phenol-NGF group and phenol-saline group, phenol-Ang II group or phenol-Ang II-losartan-PD123,319 group but not phenol-Ang II-losartan group. Also, there were significant differences in the densities of NPY-LI nerve fibers between the phenol-NGF group and phenol-saline group, phenol-Ang II group or phenol-Ang II-losartan group but not phenol-Ang II-losartan-PD123,319 group.

The relationships between the numbers of CGRP-LI and NPY-LI nerve fibers, which were counted visually, 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 all groups. There were significant positive correlations between the density and the numbers of CGRP-LI and NPY-LI nerve fibers on phenol + NGF group (CGRP-LI, $p<0.01$ $r = 0.715$; NPY-LI, $p<0.05$, $r = 0.632$) (data not shown).

Changes in vasoconstrictor responses to PNS or bolus injection of NE

Fig. 6A shows typical vascular responses induced by PNS and vasoactive agents in a perfused mesenteric vascular preparation from a

Sham rat. As shown in Fig. 6a, PNS (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 α1-adrenoceptor antagonist (prazosin) and adrenergic neuron blocker (guanethidine) (data not shown), indicating that the response was mediated by NE released due to stimulation of periarterial adrenergic nerves. Bolus injections of NE at concentrations of 5 and 10 nmol also caused concentration-dependent vasoconstriction (Fig. 6b), which was blocked by prazosin but not guanethidine (data not shown), indicating that the response was mediated by stimulation of postsynaptic α1-adrenoceptors.

As shown in Fig. 6B and Fig. 7A, the vasoconstrictor responses to PNS at 12 Hz but not 8 Hz in phenol-saline group were significantly smaller than those in the Sham group. Administration of Ang II after phenol treatment significantly increased the PNS-induced vasoconstrictor responses at 8 and 12 Hz compared with phenol-saline group. Combined administration of Ang II and losartan significantly decreased the PNS-induced vasoconstriction in the Phenol-Ang II group and addition of PD123,319 in Ang II and losartan caused a further significant decrease in the vasoconstrictor responses to PNS (8 and 12 Hz) in phenol-Ang II group. No significant change was found between the PNS-induced responses in phenol-NGF group and sham group.

Vasoconstrictor response to exogenous NE injection (5 nmol) in phenol-Ang II groups was significantly greater than those in sham group and phenol-Ang II group. The increased vasoconstrictor response to NE injection at 5 nmol but not 10 nmol was inhibited by coadministration of pheno-l-Ang II-losartan and PD123,319. The vasoconstriction induced by 10 nmol NE injection in phenol-Ang II-losartan group was greater than

those in sham, phenol-saline, phenol-Ang II-losartan-PD123,319 and phenol-NGF groups. However, significant difference was found between the phenol-Ang II-losartan group and phenol-NGF group (Fig. 7B).

Changes in vasodilator responses to PNS or bolus injection of CGRP

To observe vasodilation, active tone of the mesenteric artery was produced by continuous perfusion of 7 μM methoxamine (α1-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, as shown in Fig. 6c. The vasodilator response to PNS has been shown to be mediated by CGRPergic nerves, since the response was blocked by CGRP receptor antagonist (CGRP(8-37)) and CGRP depletor (capsaicin) (Kawasaki et al., 1990a). Bolus injections of CGRP also induced concentration-dependent vasodilation (Fig. 6d), which has been shown to be mediated by postsynaptic CGRP receptors (Kawasaki et al., 1990a).

The vasodilation in response to PNS at 2 and 4 Hz but not 1 Hz in phenol-saline, phenol-Ang II and phenol-Ang II-Losartan-PD123,319 groups but not in phenol-Ang II-losartan and phenol-NGF groups was significantly smaller than that in the sham group (Fig. 6B and Fig. 8A). The PNS (2 Hz)-response in phenol-NGF group was significantly greater than that in phenol-saline group.

As shown in Fig. 8B, vasodilation induced by exogenous CGRP injection at 25-100 pmol did not significantly alter after various treatments except phenol-NGF treatment. The vasodilation induced by 50 and 100 pmol CGRP but not 25 pmol in phenol-NGF group was significantly smaller than those in sham group, phenol-Ang II-losartan group and

phenol-saline group (100 pmol CGRP).

Western blot analysis

To investigate changes in AT2 receptor expression at Day 7 after topical phenol treatment, Western blot analysis was performed. As shown in Fig. 9, AT2 receptors, which were detected as membrane proteins of 45 kDa molecular weight, were detected in the DRG of the Sham group. Saline or Ang II administration after topical phenol treatment did not affect the AT2 receptor expression. Combined treatment with Ang II and losartan after phenol treatment tended to decrease or increase the AT2 receptor expression to less than the control level. Combination of Ang II, losartan and PD123,319 treatment after phenol treatment tended to increase the AT2 receptor expression to greater than the control level. However, no significant difference was found between the expression in the phenol-saline group and phenol-Ang II-losartan group or phenol-Ang II-losartan-PD123,319 group. In the phenol-NGF-treated group, AT2 receptor expression was significantly greater than that in the Sham group.

DISCUSSION

The present study is the first to demonstrate that activation of AT2 receptors facilitates reinnervation of perivascular CGRP-LI nerves, but not NPY-LI nerves, in the rat mesenteric artery, that was lesioned by topical application of phenol. Our recent report showed evidence that topical treatment with phenol around the rat superior mesenteric artery induced a marked reduction of innervation of perivascular NPY- and CGRP-containing nerves in the distal small artery (Hobara et al., 2006). Additionally, in this report, we have shown evidence that NGF treatment

for 7 days immediately after phenol application restored the reduction of innervation of perivascular CGRP-LI and NPY-LI nerves to the control level (Hobara et al., 2006). These findings were further confirmed by the finding in the present study that topical phenol application reduced the density of perivascular NPY-LI and CGRP-LI nerve fibers in the mesenteric artery, and these reductions were prevented by NGF treatment.

In the present study, the selective AT1 receptor blocker losartan was used to selectively stimulate AT2 receptors, since administration of Ang II in the presence of losartan results in stimulation of only AT2 receptors due to blockade of AT1 receptors. This notion was supported by the present finding that continuous infusion of Ang II caused an increase in the SBP of phenol-treated rats, and this hypertensive effect was completely eliminated by the addition of losartan, suggesting that Ang II could not stimulate AT1 receptors in the presence of losartan but that the peptide might stimulate AT2 receptors. This AT2 receptor stimulation employed in the present study resulted in a significant increase in the innervation of CGRP-LI nerves that had been markedly reduced by topical phenol treatment, whereas AT2 receptor stimulation did not affect the phenol-induced decrease in the NPY-LI nerve innervation. Furthermore, the facilitatory effect of AT2 receptor stimulation on the innervation of CGRP-LI nerves was abolished by the addition of the AT2 receptor antagonist together with Ang II and losartan, suggesting the involvement of AT2 receptors in the regeneration and/or redistribution of lesioned CGRP-LI nerve fibers. Thus, it is very likely that AT2 receptors play an important role in the reinnervation of perivascular CGRP-containing nerves that have been damaged by phenol application.

CGRP is well known to be a vasodilator neurotransmitter (Kawasaki et al., 1988). When perivascular CGRP nerves are electrically stimulated,

neurotransmitter CGRP is released from the nerves and activates CGRP receptors on vascular smooth muscle cells, causing vasodilatation (Kawasaki et al., 1988, 1990a). As shown in the present study, the PNS-induced vasodilatation of the precontracted mesenteric artery under the blockade of adrenergic neurotransmission results from CGRP released from CGRP nerves that innervate the mesenteric artery. The present finding that the vasodilator response to PNS decreased after phenol treatment was likely due to the loss of CGRP-LI nerve fibers. Combined treatment with Ang II and losartan (phenol-Ang II-losartan-treated group), which could stimulate AT2 receptors, caused greater vasodilator responses to 2 and 4 Hz PNS than those in the phenol-saline and phenol-Ang II groups and resulted in responses that were not significantly different from those in the Sham group, while the responses in the phenol-saline and phenol-Ang II-treated group were significantly smaller than that in the Sham group. Furthermore, the addition of AT2 receptor antagonist PD123,319 along with Ang II and losartan treatment caused significantly decreased vasodilatation in response to PNS, to a level similar to that in the phenol-saline-treated group. Taken together, these findings suggest that AT2 receptors have the ability to facilitate the process of reinnervation of CGRP-LI nerve fibers.

In the present study, the AT1 and AT2 receptor agonist Ang II caused a slight (but not significant) increase in the density of NPY-LI and CGRP-LI nerve fibers in the phenol-treated mesenteric artery. Lucius et al. (1998) reported that treatment with Ang II or Ang II plus losartan induces nerve regeneration in the retinal ganglion cells. However, in the present study, AT2 receptor stimulation following phenol treatment resulted in maintenance of a level similar to the density of NPY-LI nerve fibers and adrenergic (NPY-containing) nerve-mediated vasoconstriction in response

to PNS in the phenol-lesioned group, while the stimulation facilitated reinnervation of CGRP-LI nerves. These findings suggest that AT2 receptors may act to inhibit reinnervation of NPY-LI nerve fibers. This may explain why Ang II alone could not facilitate the reinnervation of NPY-LI nerve fibers in the phenol-treated mesenteric artery. Furthermore, based on the present findings, the following two possibilities should be considered; 1) stimulation of AT1 receptors inhibits reinnervation of CGRP-LI nerves and stimulation of both AT1 and AT2 receptors by Ang II produces functions to an equal extent in opposite directions each other; 2) stimulation of AT1 receptors produces no effect on reinnervation of CGRP-LI nerves, but AT1 receptors are abundantly expressed or have higher affinity to Ang II, compared to AT2 receptors.

NPY has been shown to be co-localized with the neurotransmitter NE in the granules of adrenergic nerves (Fried et al., 1985). Ang II evokes the release of NPY and NE, thereby facilitating adrenergic neurotransmission (Claudia et al., 2003). In contrast, Ang II inhibits the release of CGRP, thereby inhibiting neurotransmission in CGRP-containing nerves (Kawasaki et al., 1998). Therefore, it seems likely that the augmented vasoconstrictor response to PNS in Ang II-treated group was due to the facilitatory effect of Ang II on adrenergic nerve function.

Studies with Western blot and RT-PCR analysis have shown that AT2 receptors are localized in endothelial cells and smooth muscle cells in rat mesenteric arteries (Matrougui et al., 1999) and skeletal muscle arterioles (Nora et al., 1998). The AT2 receptors in the central nervous system (CNS) have been reported to be distributed in the hippocampus, limbic structures, thalamic area and hypothalamic areas (Reagan et al., 1994). Additionally, mRNA expression of AT2 receptors has been detected in the lateral septum, several thalamic nuclei and the inferior olive in the rat adult

brain (Lenkei et al., 1996). Furthermore, the mRNA of AT2 receptors in the lateral septum of the rat adult brain is expressed in neurons rather than glial cells (Lenkei et al., 1996). In the present study, Western blot analysis showed that AT2 receptors were detected in the DRG, which contains the cell bodies of the sensory afferent neurons and is a prominent site of CGRP synthesis. Thus, these findings support the notion that stimulation of AT2 receptors facilitates reinnervation of CGRP-LI nerves. The different effects of AT2 receptor stimulation on the reinnervation of CGRP-LI and NPY-LI nerves may be due to the different distributions of AT2 receptors. Nap et al. (2003) reported that Ang II enhances sympathetic neurotransmission via AT1 receptors located on sympathetic nerve terminals, but AT2 receptors did not enhance sympathetic neurotransmission in vitro. Therefore, it is assumed that the sympathetic ganglion may have a lower density of AT2 receptors. In the present study, decreased expression of AT2 receptors was observed in the DRG after phenol-Ang II-losartan treatment, which stimulates AT2 receptors, suggesting down-regulation of the expression of AT2 receptors due to their continuous stimulation. In contrast, blockade of AT2 receptors in Phenol-Ang II-Losartan-PD123,319 group tended to increase AT2 receptor expression, although no significance was found. These results suggest that stimulation and/or blockade of AT2 receptor function may result in down- or up-regulation of AT2 receptor expression in DRG. Furthermore, it seems likely that stimulation of AT1 receptors causes the increase in expression of AT2 receptors, which counteract down-regulation induced by stimulation of AT2 receptors. This may explain the finding that Ang II treatment alone tended to increase the expression of AT2 receptors. Since AT1 receptors are abundantly distributed, Ang II activates predominantly AT1 receptors to mask the function of AT2 receptor. In accord with this

notion, it has been reported that Ang II-infused rats showed an increase in the mRNA of vascular AT2 receptors, but coadministration of Ang II with valsartan, an AT1 receptor blocker, decreased the AT2 receptor mRNA expression (Bonnet et al., 2001). Therefore, it seems likely that the AT2 receptor expression may be correlated with the presence of AT1 receptors.

It should be noted that treatment with NGF significantly increased AT2 receptor protein expression in the DRG of phenol-treated rats. Since NGF treatment could restore the phenol-induced reduction of the density of CGRP-LI and NPY-LI nerve fibers to the control level, it is very likely that AT2 receptors play a critical role in the action of NGF. NGF has been shown to regulate the mRNA expression of AT2 receptors in neurons derived from the neonatal rat brainstem and hypothalamus (Huang et al., 1997). Additionally, it was reported that AT2 receptors promote neuronal differentiation (Lucius et al., 1998). On the other hand, AT2 receptors induce apoptosis to inactivate Bcl-2, which is an antiapoptotic protein (Horiuchi et al., 1997). Therefore, further studies are needed to clarify whether an increase of AT2 receptor expression is responsible for the neurotrophic and neuroprotective effects of NGF.

In conclusion, the present study suggests that AT2 receptors play an important role in the process of regeneration of CGRPergic nerves, which innervate mesenteric resistance arteries of the rat. We have reported that CGRPergic nerve innervation in SHR decreases with age and that long-term administration of AT1 receptor antagonist in SHR prevents the age-related decreases in function and distribution of CGRPergic nerves (Kawasaki et al., 1990b, Hobara et al., 2005). Therefore, we hypothesize that AT1 receptor antagonists may exert hypotensive effects not only by inhibiting the AT1 receptor-mediated effect of Ang II but also by activating AT2 receptors to prevent remodeling of CGRPergic nerves and facilitate

reinnervation of CGRPergic nerve fibers.

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Figure legends

Fig. 1. Changes in systolic blood pressure following topical phenol treatment. Numbers of animals in each group were as follows: Sham (n = 6); Phenol + saline (n = 5); Phenol + Angiotensin II (750 ng/kg/min) (n = 7); Phenol + Angiotensin II + losartan (0.025% dissolved in drinking water) (n = 7); Phenol + Angiotensin II + losartan + PD123,319 (10 mg/kg/day) (n = 5). Data are mean ± S.E.M. *****$p<0.05$ vs. Sham control. $^{\dagger}p<0.05$ vs Phenol + Ang II group

Fig. 2. Representative confocal laser micrographs showing changes in the density of calcitonin gene-related peptide (CGRP)-like immunoreactive (LI)-containing nerve fibers in mesenteric arteries 7 days after topical phenol treatment and administration of saline (saline), angiotensin II (Ang II), coadministration of angiotensin II with losartan (Ang II + Los), coadministration of angiotensin II with losartan and PD123,319 (Ang II + Los +PD) or nerve growth factor (NGF). The horizontal bar in the right lower corner of each panel indicates 100 μm.

Fig. 3. Changes in density of CGRP-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery and administration of saline (saline) (n = 5), angiotensin II (Ang II) (n = 7), coadministration of angiotensin II with losartan (Ang II + Los) (n = 7), coadministration of angiotensin II with losartan and PD123,319 (Ang II + Los +PD) (n = 5), or nerve growth factor (NGF) (n = 5). Sham (n = 6) indicates treatment of the superior mesenteric artery with saline instead of phenol. *****$p<0.01$ vs. Sham control. †$p<0.05$ vs. phenol-NGF. Each bar indicates mean ± S.E.M.

Fig. 4. Representative confocal laser micrographs showing changes in the density of neuropeptide Y (NPY)-like immunoreactive (LI)-containing nerve fibers in mesenteric arteries 7 days after topical phenol treatment and administration of saline (saline), angiotensin II (Ang II), coadministration of angiotensin II with losartan (Ang II + Los), coadministration of angiotensin II with losartan and PD123,319 (Ang II + Los +PD) or nerve growth factor (NGF). The horizontal bar in the right lower corner of each panel indicates 100 μm.

Fig. 5 Changes in density of neuropeptide Y (NPY)-like immunoreactive (LI)-containing nerve fibers in distal mesenteric arteries after topical phenol treatment of the superior mesenteric artery and administration of saline (saline) (n = 5), angiotensin II (Ang II) (n = 7), coadministration of angiotensin II with losartan (Ang II + Los) (n = 7), coadministration of angiotensin II with losartan and PD123,319 (Ang II + Los +PD) (n = 5), or nerve growth factor (NGF) (n = 5). Sham (n = 6) indicates treatment of the superior mesenteric artery with saline instead of phenol. ***p*<0.01 vs Sham control. †*p*<0.05 vs. phenol-NGF. Each bar indicates mean ± S.E.M.

Fig. 6. Typical records showing vasoconstrictor responses induced by periarterial nerve stimulation (PNS) and bolus infusion of norepinephrine (NE, 5 and 10 nmol) and vasodilator responses induced by PNS and bolus injection of calcitonin gene-related peptide (CGRP, 25, 50 and 100 pmol) in perfused mesenteric vascular beds. A; the preparation isolated from a Sham control rat. B; vasoconstrictor and vasodilator responses in a preparation isolated from a phenol-saline-treated rat. C; vasoconstrictor and vasodilator responses in a preparation isolated from a

phenol-angiotensin II (Ang II)-losartan (Los)-treated rat. a and b; vasoconstrictor responses in the preparation with resting tone. c and d: vasodilator responses in the preparation with active tone produced by 7 μM methoxamine in the presence of 5 μM guanethidine. PPV; perfusion of papaverine.

Fig. 7. Changes in vasoconstrictor responses to perivascular nerve stimulation (PNS) and to bolus infusion of norepinephrine (NE) in perfused mesenteric vascular beds isolated from the Sham control (n = 7), Phenol + saline (n = 12), Phenol + Angiotensin II (Ang II; 750 ng/kg/day) (n= 6), Phenol + Angiotensin II (Ang II) + losartan (Los; 0.025 % dissolved in drinking water) (n = 7), Phenol + Angiotensin II (Ang II) + losartan (Los) + PD123,319 (PD; 10 mg/kg/day) (n = 5) and Phenol + NGF (NGF; 20 μg/kg/day) (n = 4) groups. *****p<0.05 vs. Sham control. Each bar indicates mean ± S.E.M.

Fig. 8. Changes in vasodilator responses to perivascular nerve stimulation (PNS) and to bolus infusion of calcitonin gene-related peptide (CGRP) in perfused mesenteric vascular beds isolated from group of Sham control (n = 10), Phenol + saline (n = 12), Phenol + Angiotensin II (Ang II; 750 ng/kg/day) (n= 6), Phenol + Angiotensin II (Ang II) + losartan (Los; 0.025 % dissolved in drinking water) (n = 7), Phenol + Angiotensin II (Ang II) + losartan (Los) + PD123,319 (PD; 10 mg/kg/day) (n = 5) and Phenol + NGF (NGF; 20 μg/kg/day) (n = 4) groups. The active tone was produced by 7 μM methoxamine in the presence of 5 μM guanethidine. Relaxation is expressed as the percentage of maximum relaxation induced by perfusion of 100μM papaverine at the end of each experiment. *****p<0.05 vs. Sham control. Each bar indicates mean ± S.E.M.

Fig. 9. Western blot analysis of angiotensin type 2 receptor (AT2 receptor) protein expression in dorsal root ganglia (DRG) isolated from the Sham control (n = 3), Phenol + saline (n = 3), Phenol + nerve growth factor (NGF) (n = 3), Phenol + Angiotensin II (Ang II) + losartan (Los) (n = 3**)**, Phenol + Ang II + losartan + PD123,319 (PD) (n = 3**)** and Phenol + Ang II (n = 4) groups. Molecular weight of the AT2 receptor and β-actin proteins indicated in the upper right was about 45 kDa and 47 kDa, respectivdely. *****p<0.05 vs. Sham control. The ratio of the level of each test protein to β-actin was determined by densitometric analysis. Each bar indicates mean ± S.E.M.