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Proton acts as a neurotransmitter for nicotine-induced adrenergic and CGRPergic nerve-mediated vasodilation in the rat mesenteric artery

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Abbreviations:

ACh, acetylcholine; **CGRP**, calcitonin gene-related peptide; **CGRPergic**, calcitonin gene-related peptide (CGRP)-containing; **COMT**,

catechol-o-methyl transferase; **HCl** , hydrochloric acid; **L-DOPA**, 3,4-dihydroxyphenylalanine; **LI**, like immunoreactivities; **MAO**, monoamine oxidase; **NANC**, nonadrenergic noncholinergic; **NPY**, neuropeptide Y; **NO**, nitric oxide; **PBS**, phosphate-buffered saline; **PNS**, periarterial nerve stimulation; **SD**, sodium deoxycholate; **TH,** tyrosine hydroxylase; **TRPV1**, transient receptor potential vanilloid-1

Abstract

Nicotine stimulates presynaptic nicotinic acetylcholine receptors in perivascular adrenergic nerves and release unknown transmitter(s), which activate transient receptor potential vanilloid-1 (TRPV1) located on calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves, resulting in vasodilation. The present study investigated a potential transmitter transmitting between perivascular adrenergic nerves and CGRPergic nerves. Rat mesenteric vascular beds without endothelium were contracted by perfusion with Krebs solution containing methoxamine and perfusion pressure and pH levels of perfusate were measured. Nicotine perfusion for 1 min induced concentration-dependent vasodilation and lowered pH levels, which were abolished by cold-storage denervation of preparations, guanethidine (adrenergic neuron blocker) and mecamylamine (nicotinic $\alpha_3\beta_4$ acetylcholine receptor antagonist). Capsazepine (TRPV1 antagonist) blunted nicotine-induced vasodilation, but had no effect on pH lowering. Injection of hydrochloric acid (HCl) and perfusion of Krebs solution at low pH (6.0 to 7.2) induced vasodilation. HCl-induced vasodilation was inhibited by cold-storage denervation, capsazepine, capsaicin (CGRP depletor) and CGRP(8-37) (CGRP receptor antagonist). Perfusion of adrenergic transmitter metabolites (normetanephrine and 3-methoxydopamine), but not other metabolites, induced vasodilation, which was not inhibited by capsaicin treatment. Immunohistochemical staining of mesenteric arteries showed dense innervation of CGRP- and TRPV1-immunopositive nerves, with both immunostainings appearing in

the same neuron. Mesenteric arteries were densely innervated by neuropeptide Y (NPY)-immunopositive nerves, which coalesced with CGRP-immunopositive nerves. Scanning and immunoscanning electron microscopic images showed coalescence sites of different perivascular fibers before they intruded into smooth muscles. These results indicate that nicotine initially stimulates adrenergic nerves via nicotinic $\alpha_3\beta_4$ receptors to release protons and thereby induces CGRPergic nerve-mediated vasodilation via TRPV1.

Introduction

It is widely accepted that the vascular tone is mainly maintained by sympathetic adrenergic nerves via the release of the neurotransmitter norepinephrine. However, accumulating evidence reveals that nonadrenergic, noncholinergic (NANC) vasodilator nerves also play a role in regulation of vascular tone. We have demonstrated that the rat mesenteric artery has dense innervation of calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves, which release a transmitter, CGRP, causing vasodilation (Kawasaki *et al.*, 1988). Recently, we reported that nitric oxide (NO)-containing nerves innervating rat mesenteric arteries are involved in modulation of adrenergic neurotransmission (Hatanaka *et al*. 2006). The rat mesenteric artery is also densely innervated by adrenergic nerves, which contain the main neurotransmitter, norepinephrine, with a neuropeptide Y (NPY). Observations of double immunostainings in immunohistochemical studies showed the appearance of both NPY- and CGRP-immunoreactivities in the same neurons of the rat mesenteric artery (Eguchi *et al*. 2004), leading to a hypothesis for some interactions between adrenergic nerves and CGRPergic nerves.

Our previous reports showed evidence that a functional deficiency in CGRPergic nerves augments adrenergic nerve-mediated vasoconstriction, and conversely, adrenergic nerves via norepinephrine release presynaptically inhibit the neurogenic release of CGRP from the nerve, decreasing CGRPergic nerve function (Kawasaki *et al.*, 1990a; 1990b).

Thus, we have proposed that CGRPergic vasodilator nerves and sympathetic vasoconstrictor nerves reciprocally regulate the tone of the mesenteric resistance artery. Our recent studies revealed evidence that nicotine, a nicotinic acetylcholine (ACh) receptor agonist, induces perivascular adrenergic nerve- and CGRPergic nerve-mediated vasodilation in the rat mesenteric artery (Shiraki *et al*., 2000; Eguchi *et al*., 2004; Eguchi *et al*., 2007). In these studies, we proposed a hypothesis that nicotine first stimulates nicotinic ACh receptors located on adrenergic nerves to release transmitter(s), that activate adjacent CGRPergic nerves through transient receptor potential vanilloid 1 (TRPV1) to induce CGRP-mediated vasodilation. Based on these studies, it is expected that nicotine releases an endogenous agonist for TRPV1 receptors from adrenergic nerves (Eguchi *et al*. 2004). TRPV1 receptors have been shown to be stimulated by protons (H^+) (Tominaga and Tominaga, 2005), anandamide (Zygmunt et al., 1999) and lipids metabolites (Van der Stelt and Di Marzo, 2004). However, the substance(s) transmitting axo-axonal information from perivascular adrenergic nerves to CGRPergic nerves remain unclear.

Therefore, the present study focused on possible endogenous agonist transmitters for TRPV1 involved in axo-axonal transmission of adrenergic nerves and CGRPergic nerves.

Methods

Animals. Male Wistar rats weighing 250-350 g were used in the present study. All animals were given food and water *ad libitum*. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22 ± 2 ºC with 50 ± 10% relative humidity and a 12-h light/12-h dark cycle (light on 08:00 a.m.). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 115) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and their suffering.

Perfusion of the mesenteric vascular beds. The animals were anesthetized with pentobarbital-Na (50 mg/kg, intraperitoneally) and the mesenteric vascular beds were isolated and prepared for perfusion as described previously (Kawasaki *et al.*, 1988; Kawasaki *et al.*, 1990a; Shiraki *et al*., 2000). Briefly, the superior mesenteric artery was cannulated and the entire intestine and associated vascular bed was isolated. 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. The isolated mesenteric vascular bed was then placed in a water-jacketed organ bath

maintained at 37 ºC and perfused with a modified (see below) 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_2-5 % CO_2 before passage through a warming coil maintained at 37ºC. 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; disodium EDTA 0.03 and dextrose 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).

In the series of experiments using Krebs solution at different pH levels, Krebs solution at pH 7.2, 7.0, 6.5 and 6.0 was prepared by reducing concentration of $NaHCO_3$ (pH 7.4, 20 mM) to 15 mM, 5 mM, 3 mM and 1 mM, respectively, and by increasing NaCl (pH 7.4, 120 mM) to 130 mM, 140 mM, 142 mM and 144 mM, respectively. The Krebs solution at different pH levels containing methoxamine (1.5-2 μM) was perfused for 1 min.

Chemical removal of the vascular endothelium. The preparations with resting tone were perfused with a 1.8 mg/ml solution of sodium deoxycholate (SD) in saline for 30 s to remove the vascular endothelium, as described by Takenaga *et al.* (1995) and Shiraki *et al*. (2000). Then, the

preparations were rinsed with SD-free Krebs solution for 60 min. After the preparations were contracted by perfusion with Krebs solution containing methoxamine (2 μM), successful removal of the endothelium was assessed by the lack of a relaxant effect after a bolus injection of 1 nmol acetylcholine (ACh), which was injected directly into the perfusate proximal to the arterial cannula with an infusion pump (model 975, Harvard Apparatus, Holliston, MA., USA.). The volume injected was 100 μl over 12 s.

Periarterial nerve stimulation (PNS). PNS was applied for 30 s using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 msec and a supramaximal voltage (50 V) were applied at 2 Hz using an electronic stimulator (model SEN 3301, Nihon Kohden).

In vitro treatment with capsaicin. In vitro depletion of perivascular CGRP-containing nerves was performed according to the method described by Kawasaki *et al.* (1988; 1990a). The preparation was perfused with Krebs solution containing capsaicin (CGRP depletor) (1 μM) for 20 min and then rinsed for 60 min with capsaicin-free Krebs solution. After the endothelium was removed with SD perfusion, the preparation was contracted by perfusion of Krebs solution containing methoxamine (2 μM). After the elevated perfusion pressure stabilized, a bolus injection of acetylcholine (1 nmol) was performed to check for the presence of the endothelium, and

then PNS at 2 Hz was performed to check for the presence of CGRPergic nerves. Successful depletion of CGRPergic nerves was confirmed by the lack of a relaxant effect induced by periarterial nerve stimulation (2 Hz).

Cold-storage denervation. The isolated mesenteric vascular bed was stored in cold Krebs solution at 4°C for 72 h to achieve cold-storage denervation, as described previously (Kawasaki *et al*., 1991; Shiraki *et al*., 2000; Hobara *et al*., 2004). After being perfused with Krebs solution at 37°C for 60 min, the preparation was subjected to the experiment. After removal of endothelium by SD perfusion, the preparation was contracted by perfusion of Krebs solution containing methoxamine (α_1-adrenoceptor agonist). The concentration of methoxamine was increased to 5-7 μM, since the cold-storage treatment decreased vasoconstrictor reactivity to methoxamine. To determine the vascular smooth muscle activity, a bolus injection of CGRP (50 pmol) was given to cause vasodilation. Successful denervation of periarterial nerves was confirmed by the lack of PNS-induced vasoconstriction (8 and 12 Hz) at resting tone, and vasodilation (2 Hz) at active tone.

Measurement of pH levels in the perfusate. The isolated mesenteric vascular bed was perfused with Krebs solution. A pH sensor was placed around the apical portion of each preparation and changes in the pH value of the perfusate, which had flowed out from the preparation, were continuously monitored with the pH meter (model F-54, HORIBA Co.,

Kyoto, Japan). Changes in pH levels were expressed as a percent of pH values before perfusion of Krebs solution containing the agents tested.

Measurement of norepinephrine in the perfusate. The isolated mesenteric vascular bed was perfused with Krebs solution and the perfusate was collected before and after perfusion of nicotine for 3 min in the absence (control) or presence of guanethidine. Norepinephrine in the perfusate was adsorbed onto alumina and eluted with acetic acid. The eluate was assayed by high-performance liquid chromatography with electrochemical detection (model HTEC-500, Eicom, Kyoto, Japan). The internal standard was dihydroxybenzylamine.

Perfusion of nicotine and pharmacological analysis. After chemical removal of the endothelium, the preparations were perfused with Krebs solution containing methoxamine at a concentration of 2 μM to induce submaximal vasoconstriction. After stabilization of the elevated perfusion pressure, Krebs solution containing final concentrations of nicotine at 1, 10 or 100 μM and methoxamine was perfused for 1 min as the control response and then Krebs solution containing methoxamine and guanethidine (adrenergic neuron blocker) (5 μM), capsazepine (TRPV1 antagonist) (10 μM) or mecamylamine (nicotinic$\alpha_3\beta_4$ ACh receptor antagonist) (10 μM) were perfused. Then, after the elevated perfusion pressure had stabilized, Krebs solution containing the final concentration of nicotine and guanethidine, capsazepine or mecamylamine was perfused for

1 min. In the experiments using capsazepine (10 μM), the concentration of methoxamine was increased to 10 μM, since these antagonists caused vasodilation and weak active tone. To avoid tachyphylaxis, the perfusion of nicotine was carried out at 20-min intervals.

In the series of experiments using HCl, bolus injections of 0.1 mol HCl were given during perfusion of capsazepine (10 μM), CGRP(8-37) (0.1 μM; CGRP receptor antagonist) and also given to in preparations treated with capsaicin (1 μM) or preparations with cold-storage denervation.

In the series of experiments using various catecholamine metabolites, Krebs solution containing methoxamine and a concentration of catechol-O-methyl transferase (COMT) metabolite of dopamine (3-methoxytyramine, homovanillic acid) or norepinephrine (normetanephrine, vanillylmandelic acid) or monoamine oxidase (MAO) metabolite of dopamine (3,4-dihydroxyphenyl acetic acid) or norepinephrine (3,4-dihydroxymandelic acid) was perfused for 1 min in rat-denuded mesenteric vascular beds treated with or without capsaicin.

At the end of each experiment, the preparations were perfused with 100 μM papaverine to induce complete relaxation. Vasodilator activity is expressed as the percentage of perfusion pressure at the maximum relaxation induced by papaverine.

Immunohistochemical Study

Confocal laser microscopy. Under pentobarbital-Na anesthesia, the mesenteric artery with the intestine was surgically removed and the third

order branch of the artery was isolated and fixed in Zamboni's fixative containing 2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer for 48 h. After fixation, the specimens were rinsed repeatedly in phosphate-buffered saline (PBS). The mesenteric artery was immersed in PBS containing 0.5% Triton X-100 overnight, followed by incubation with PBS containing normal goat serum (1:100) for 60 min. The tissues were then incubated with the primary antibody: anti-TRPV1 (1:10,000; raised in rabbit) (Affinity Bio Regents, Golden, CO, USA) or anti-NPY (1:300; raised in rabbit) (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), for 72 h at 4ºC. After incubation, the tissues were washed in PBS and the site of the antigen-antibody reaction was revealed by incubation with fluorescein-5-isothiocyanate-labeled goat anti-rabbit IgG (diluted 1:100) (ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 60 min. The tissues were thoroughly washed in PBS, mounted in glycerol/PBS 2:1 (v/v) and observed under a confocal laser scanning microscope (CLSM 510, Carl Zeiss, Germany). Control immunohistochemical staining for TRPV1 was done by preabsorbing the blocking peptide for the primary antibody to TRPV1 (rat) (20 µg/ml; Alexis Biochemicals, San Diego, CA, USA) and exhausting the TRPV1 antibody with the relevant peptides.

Double immunostainings. Mesenteric arteries were incubated with PBS containing 1% normal goat serum, 1% bovine serum albumin and 0.03% Triton X-100 for 1 h. Then, the sections were incubated with an anti-TRPV1 antibody (1:10,000; raised in rabbit) or anti-NPY antibody

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(1:300; raised in rabbit) (Phoenix Pharmaceuticals, Inc.) at 4ºC for 2 days, followed by incubation with an anti-CGRP antibody (1:300; raised in guinea pig) at 4ºC for 2 days. Following washes in PBS, the sections were incubated for 2 h with rhodamine-conjugated goat $F(ab')_2$ fraction anti-guinea pig IgG (diluted 1:100) (ICN Pharmaceuticals, Inc.) at 4ºC, followed by incubation with fluorescein-5-isothiocyanate-conjugated rabbit IgG fractions (diluted 1:100) (ICN Pharmaceuticals, Inc.) at 4ºC for 1 h. The samples were then observed under a confocal laser scanning microscope (CLSM 510, Carl Zeiss) with an exciting filter system (458/488 nm for FITC) and emission filter system (543 nm for rhodamine). Two fluorescence views were obtained from the same microscopic field..

Scanning Electron microscopic study.

Tissue processing. Under pentobarbital-Na anesthesia, mesenteric arteries were isolated, excised, fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C, then rinsed in 0.1 M phosphate buffer for 10 min and treated with 30 % KOH solution at 60˚C for 10 min to dissolve connective tissue matrices around vessels. The specimens were immersed in 0.5 % tannic acid solution for 1 h and then postfixed with 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 h. The samples were rinsed with 0.1 M phosphate buffer and then dehydrated through a graded series of ethanol and dried with *t*-butyl alcohol using a critical-point drier. They were observed in a scanning electron microscope (S-2300, Hitachi, Tokyo, Japan) under an accelerating voltage of 25 kV.

Immunoscanning electron microscopy: Under pentobarbital-Na anesthesia, the second-third order branches of the mesenteric arteries were isolated, dissected out and placed in Zamboni's fixative containing 2% paraformaldehyde and 15% picric acid in 0.15 mol/L phosphate buffer for 48 h. After fixation, the arteries were rinsed repeatedly with PBS, immersed in PBS containing 0.5% Triton X-100 for 48 h, and then incubated with PBS containing normal goat serum (1:100) for 60 min. The arteries were then incubated with the primary antibodies: anti-tyrosine hydroxylase (rate-limiting enzyme of tyrosine in adrenergic nerves) (TH; 1:300; raised in rabbits; Phoenix Pharmaceuticals, Inc.) or anti-CGRP (1:500; raised in rabbits; BIOMOL International, LP, Plymouth Meeting, PA, USA) for 72 h at 4°C. After incubation, the arteries were washed with PBS and incubated for 30min with a horseradish peroxidase conjugated secondary antibody (1:100; R&D Systems, Inc., Minneapolis, USA) for 3 h at 4°C. The antigen-antibody complex was then visualized by incubating the arteries with 3, 3'-diaminobenzidine solution (3, 3'-diaminobenzidine 12 mg/mL, 1 mL Tris-HCl at pH7.4 and 3% H_2O_2) for 15 to 30 min. The arteries were then fixed with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C, rinsed in 0.1 M phosphate buffer for 10 min and treated with 30 % KOH solution at 60°C for 10 min to dissolve connective tissue matrices around vessels. The specimens were immersed in 0.5 % tannic acid solution for 1 h and then postfixed with 1 % osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h. The samples were

washed with phosphate buffer, dehydrated through a graded series of ethanol and dried with *t*-butyl alcohol using a critical-point drier. They were observed in a scanning electron microscope (S-900, Hitachi) under an accelerating voltage of 10 or 15 kV.

Statistical analysis.

Experimental results are presented as the mean ± s.e.m. Statistical analysis was done using the Student's unpaired *t* test and one-way analysis of variance followed by Tukey's test. A $P < 0.05$ was considered significant.

Drugs

The following drugs were used: ACh chloride (Daichi Pharmaceutical Co., Tokyo, Japan), capsaicin (Sigma Aldrich Japan., Tokyo, Japan), capsazepine (Sigma), 3,4-dihydroxyphenyl acetic acid (Sigma), 3,4-dihydroxymandelic acid (Sigma), guanethidine sulfate (Tokyo Kasei, Tokyo, Japan), homovanillic acid (Sigma), human CGRP(8-37) (Peptide Institute, Osaka, Japan), dihydroxybenzylamine (Sigma), 3-methoxytyramine (Sigma), normetanephrine (Sigma), norepinephrine hydrochloride (Sankyo-Daiichi, Tokyo, Japan), nicotine tartrate salt (Sigma), mecamylamine (Sigma), methoxamine hydrochloride (Nihon Shinyaku Co., Kyoto, Japan), papaverine hydrochloride (Sigma), rat CGRP (Peptide Institute), SD (Ishizu Seiyaku, Tokyo, Japan) and vanillylmandelic acid (Sigma). All drugs, except for capsaicin and SD, were dissolved in pure water and diluted with Krebs solution. Capsaicin was dissolved in 50

% ethanol and diluted with Krebs solution (final alcohol concentration, 0.4 mg/ml). SD was dissolved in 0.9 % saline. ACh and rat CGRP was diluted with Krebs solution containing 2 μM methoxamine when injected directly.

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Results

Vasodilator response and pH changes induced by nicotine perfusion. As shown in Figure 1A, in the SD-treated preparation with active tone produced by methoxamine, bolus injection of ACh did not induce a vascular response, indicating that the endothelium had lost its function. PNS at 1 Hz caused an initial increase in perfusion pressure due to vasoconstriction, followed by long-lasting decrease in perfusion pressure due to vasodilation, indicating that perivascular nerve function was left intact. PNS-induced vasoconstriction and vasodilation have been shown to be mediated by adrenergic nerves and CGRPergic nerves, respectively (Kawasaki *et al.*, 1988; Takenaga *et al.*, 1995).

In this preparation, perfusion of nicotine caused a concentration-dependent vasodilation (Figure 1A). Basal pH levels of the perfusate before the first and second series of nicotine perfusion was 7.547 ± 0.061 (n=9) and 7.454 ± 0.103 (n=9), respectively. The nicotine perfusion decreased perfusate pH values (Figure 1B), which appeared during the vasodilation, started within 1 min after nicotine perfusion, reached a maximum at 5 to 7 min and then returned to pre-perfusion pH levels (Figure 1B). As shown in Figure 2, the nicotine-induced vasodilation (Figure 2A) and pH lowering (Figure 2B) was concentration-dependent and reproducible when nicotine perfusion was repeated. In some preparations, a very slight vasoconstriction by nicotine at low concentrations of 1-10 μM preceded the vasodilation, but no vasoconstriction to nicotine was observed

at higher concentrations (data not shown).

In preconstricted preparations treated with cold-storage, PNS at 2 Hz did not cause a vascular response (data not shown), indicating that function of perivascular nerves had been effectively eliminated. As shown in Figure 2C, in denervated preparations, perfusion of nicotine induced neither vasodilation nor a pH lowering (Figure 2D). Basal pH levels of the perfusate before the first series of nicotine perfusion was 7.667 ± 0.043 (n=3).

As shown in Fig. 3, the nicotine-induced vasodilation (Figure 3A) and pH lowering (Figure 3B) were abolished by guanethidine, which eliminated PNS-induced adrenergic nerve-mediated vasoconstriction, but not CGRPergic nerve-mediated vasodilation (data not shown). Basal pH levels of the perfusate before the first (control) and second (guanethidine) series of nicotine perfusion was 7.545 ± 0.089 (n=5) and 7.510 ± 0.060 (n=5), respectively.

Figure 3C and 3D show that mecamylamine markedly inhibited vasodilation and the pH lowering induced by nicotine perfusion, respectively. Basal pH levels of the perfusate before the first (control) and second (mecamylamine) series of nicotine perfusion was 7.494 ± 0.072 (n=5) and 7.502 ± 0.074 (n=5), respectively.

As shown in Figure 3E, capsazepine markedly inhibited nicotine-induced vasodilation, while the antagonist did not affect the nicotine-induced pH lowering, but rather enhanced it (Figure 3F). Basal pH levels of the perfusate before the first (control) and second (capsazepine)

series of nicotine perfusion was 7.589 ± 0.129 (n=5) and 7.450 ± 0.250 (n=5), respectively.

Vasodilator response induced by HCl (H^+) injection. In perfused mesenteric vascular beds without endothelium, as shown in Figure 4A, a bolus injection of HCl induced an initial sharp vasodilation followed by a long-lasting vasodilation (80.4 ± 6.4%, n=5), while NaCl injection had no effect (0.6 ± 0.02 %; n=3), indicating that protons (H^+), but not chloride anion (Cl^-), were responsible for the vasodilation. Bolus injection of HCl lowered pH levels of the perfusate from 7.692 ± 0.054 (basal pH level; n=3) to 7.58 ± 0.52 (1.45% decrease; n=3), while NaCl injection did not change pH levels of perfusate (basal pH, 7.693 ± 0.047; pH after NaCl injection, 7.686 ± 0.039; 0.09% decrease; n=3).

Figure 4B showed that the HCl-induced vasodilation was significantly inhibited by cold-storage denervation, capsazepine, CGRP(8-37) and capsaicin treatment.

Vasodilator response induced by perfusion of Krebs solution at different pH levels. In perfused mesenteric vascular beds without endothelium, as shown in Figure 5A, perfusion of Krebs solution at 7.2, 7.0, 6.5 or 6.0 for 1-min induced a sharp vasodilation in a pH-dependent manner (Figure 5B). However, perfusion of Krebs solution at pH 7.4 did not cause vasodilation (Figure 5).

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Norepinephrine release induced by nicotine. In rat-perfused mesenteric vascular beds with active tone, spontaneous release of norepinephrine was detected in the perfusate (273.4 ± 99 pg/ml, n=7). As shown in Figure 6, 1 min perfusion of nicotine at a concentration of 100 μM significantly increased norepinephrine levels (345.7 ± 120 pg/ml, n=7, $P<0.05$) in the perfusate 2 min after the perfusion, compared with pre-perfusion levels. Increased norepinephrine levels were observed until 10 min after nicotine perfusion. In the presence of guanethidine, spontaneous release of norepinephrine was also detected in the perfusate (183.7 ± 68 pg/ml, n=4). Guanethidine abolished the norepinephrine release induced by nicotine at 2 min after the perfusion (188.3 ± 70 pg/ml, n=4) and throughout the experiment (Figure 6).

Vascular responses to perfusion of various catecholamine metabolites. As shown in Table 1, perfusion of 3-methoxytyramine (COMT-metabolite of dopamine) and normetanephrine (COMT-metabolite of norepinephrine) induced concentration-dependent vasodilation in rat-denuded mesenteric vascular beds. However, capsaicin treatment, which abolished nicotine-induced vasodilation (Shiraki *et al.*, 2000), did not affect the vasodilation induced by the two metabolites (Table 1). As shown in Table 1, a MAO-metabolite (3,4-dihydroxyphenyl acetic acid) of dopamine and its COMT-metabolite (homovanillic acid) and MAO-metabolite (3,4-dihydroxymandelic acid) of norepinephrine and its COMT-metabolite (vanillylmandelic acid) had no vascular effect.

Immunohistochemical studies of perivascular nerves.

Immunohistochemical staining of the mesenteric artery showed dense innervation of CGRP-like immunoreactivities (LI) fibers (Figure 7A) and adrenergic NPY- LI fibers (Figure 7B). Innervation of NPY-LI fibers was greater than that of CGRP-LI fibers (Figure 7). As shown in Figure 7C and 7D, double immunostainings showed that most CGRP-LI fibers in contact with NPY-LI fibers appeared as yellow fibers when merged, indicating that there were close contact sites between adrenergic and CGRPergic nerves. Figure 7E and 6F show CGRP-LI fibers and TRPV1-LI fibers in the same area of the rat mesenteric artery, respectively. Additionally, as shown in Figure 7G, double immunostainings showed that both immunostainings appeared in the same neuron when merged.

Scanning electron microscopic study. Figure 8A shows a scanning electron microscopic image of perivascular nerve fibers in the mesenteric artery with many fibers running on vascular smooth muscles. Additionally, the image showed that several fibers or two fibers had coalescence sites (Figure 8A; asterisks) before they intruded into vascular smooth muscles (Figure 8A; arrows). Additionally, Figure 8B shows coalescenced nerve fibers (asterisk) and synapse-like contact between two different fibers (a and b) was observed in Figure 8C.

Figure 9 shows immunoscanning electron microscopic images of CGRP (Figure 9A) and tyrosine hydroxylase (TH; Figure 9B)-immunoreactive

nerve fibers, which are observed as light red colors. As shown in Figure 9A, CGRP-immunoreactivities-containing nerves were thin and linear fibers with a smooth surface, while TH-immunoreactivity-containing nerves as observed in Figure 9B were thick and winding fibers with a rough surface. Figure 9B also shows that TH-immunoreactivity-containing nerves (a) had coalescence sites (c) with different nerves (b), which did not contain immunoreactivities and were thin and linear fibers similar to the CGRP-immunoreactivity-containing fibers in Figure 9A.

Discussion

The rat mesenteric resistance artery has innervation of adrenergic vasoconstrictor nerves and NANC vasodilator nerves in which CGRP, a potent vasodilator peptide, acts as a vasodilator neurotransmitter (Kawasaki *et al*., 1988; Kawasaki *et al*., 1990a). Inhibition of CGRPergic function by capsaicin and CGRP(8-37) augmented adrenergic nerve-mediated vasoconstriction in response to field stimulation, suggesting that CGRPergic nerves suppress sympathetic nerve-mediated vasoconstriction (Kawasaki *et al.*, 1990a). Conversely, CGRPergic nerve-mediated vasodilation was blunted by α_2-adrenoceptor agonists (clonidine and norepinephrine) and NPY without affecting exogenous CGRP-induced vasodilation, suggesting that sympathetic nerves presynaptically inhibit the release of CGRP from the nerve decreasing CGRPergic nerve function (Kawasaki *et al.*, 1990b; Kawasaki *et al*., 1991). Thus, we proposed that CGRPergic vasodilator nerves along with sympathetic vasoconstrictor nerves reciprocally regulate the tone of the mesenteric resistance artery.

The present immunohistochemical study confirmed previous findings that rat mesenteric arteries were densely innervated by NPY- LI- and CGRP-LI-nerve fibers. The NPY-LI innervation has been shown to be eliminated by cold-storage denervation and 6-hydroxydopamine (adrenergic neurotoxin), but not capsaicin, while CGRP-LI fibers were abolished by cold-storage denervation and capsaicin, but not 6-hydroxydopamine (Hobara *et al*., 2004), indicating that CGRPergic nerves are capsaicin-sensitive and different from adrenergic nerves. In the

present additional study, double immunostainings showed both NPY-LI- and CGRP-LI-nerve fibers in the same neuron of the rat mesenteric artery. This finding is in accordance with results reported by Eguchi *et al*. (2004). Additionally, the scanning electron microscopic study suggests that perivascular nerves have coalescence sites, probably CGRPergic nerves and sympathetic adrenergic nerves. Furthermore, immunoscanning electron microscopic study using anti-CGRP for CGRPergic nerves and anti-TH for adrenergic nerves showed evidence that TH-immunoreactivity-containing nerves, adrenergic nerves, have coalescence sites with different nerves with different transmitters, probably CGRPergic nerves. Taken together, these findings strongly suggest that there are close contact areas between adrenergic and CGRPergic nerves and that both nerves interact at these areas.

Our previous studies showed that in rat-perfused mesenteric vascular beds with active tone and without endothelium, perfusion of nicotine and nicotinic ACh receptor agonists (cytisine and epibatidine) caused concentration-dependent vasodilation without inducing vasoconstriction (Shiraki *et al*., 2000; Eguchi *et al*., 2007). The nicotine-induced vasodilation was abolished by cold-storage denervation, suggesting that perivascular nerves are responsible for the effect of nicotine. Additionally, vasodilation induced by nicotine and nicotinic ACh receptor agonists was blunted by the adrenergic neuron blocker guanethidine and CGRP depletor capsaicin, suggesting that intact adrenergic nerves and CGRPergic nerves mediate the vasodilation (Shiraki *et al*., 2000; Eguchi *et al*., 2007).

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Furthermore, mecamylamine ($\alpha_3\beta_4$ nicotinic ACh receptor antagonist), but not α-bungarotoxin (α_7 nicotinic ACh receptor antagonist) or dihydro-β-erythroidine ($\alpha_4\beta_2$ nicotinic ACh receptor antagonist), abolished the nicotine- and nicotinic ACh receptor agonist-induced vasodilation (Eguchi *et al*., 2007). Therefore, based on these results, it can be hypothesized that nicotine initially stimulates $\alpha_3\beta_4$ nicotinic ACh receptors, which are located on adrenergic nerves, to release adrenergic neurotransmitter(s) and related substances. These released substances activate receptors located on adjacent CGRP nerves and then release CGRP, causing vasodilation.

To clarify possible substances transmitting nicotine-induced vasodilation, our previous study investigated using several antagonists, which antagonize the effect of adrenergic neurotransmitters (Eguchi *et al*., 2004). However, this study showed that the nicotine-induced vasodilation was not inhibited by L-NAME (NO synthase inhibitor), propranolol (non-selective β-adrenoceptor antagonist), 3,4-dihydroxyphenylalanine (L-DOPA) cyclohexyl ester (DOPA receptor antagonist) (Misu *et al*., 1997), SCH23390 (selective dopamine D_1 receptor antagonist), haloperidol (dopamine D_2 receptor antagonist), α,β-methylene ATP (ATP P_{2x} receptor desensitizing agonist), 8(p-sulfophenyl)theophylline (adenosine A_2 receptor antagonist) or BIBP3226 (NPY-Y_1 receptor antagonist) (Eguchi *et al*., 2004). In the present study, perfusion of 3-methoxytyramine (COMT-metabolite of dopamine) and normetanephrine (COMT-metabolite of norepinephrine), but not other MAO- and COMT-metabolites of

dopamine and norepinephrine, induced concentration-dependent vasodilation in rat denuded-mesenteric vascular beds. However, capsaicin treatment, which abolished nicotine-induced vasodilation, did not affect the vasodilation induced by either metabolite, suggesting that capsaicin-sensitive CGRPergic nerves are not responsible for vasodilation induced by the two metabolites. Taken together, it is unlikely that catecholamines, catecholamine metabolites or other transmitters, which are released from perivascular adrenergic nerves, are involved in the nicotine-induced vasodilation.

The present and previous studies with immunohistochemical staining of the mesenteric artery showed dense innervation of CGRP-LI- and TRPV1-LI-immunopositive nerves, with both immunostainings appearing in the same neuron (Eguchi *et al.*, 2004). Therefore, these results strongly suggest that CGRPergic nerves innervating mesenteric arteries have TRPV1. Furthermore, the present and previous studies demonstrated that capsazepine (selective TRPV1 antagonist) and ruthenium red (inhibitor of TRPV1 response) inhibited nicotine-induced vasodilation without affecting vasodilation in response to exogenously applied CGRP (Eguchi *et al.*, 2004), suggesting that TRPV1 is involved in the nicotine-induced vasodilation.

It is well known that the primary sensory nerves are activated by protons (H^+) via TRPV1 (Tominaga and Tominaga, 2005). In the present study, exogenously applied HCl in rat mesenteric vascular beds without endothelium resulted in endothelium-independent vasodilation associated

with pH lowering of the perfusate. Since NaCl injection had no vascular effect, H^+, but not Cl^-, is responsible for the HCl-induced vasodilation. Additionally, cold-storage denervation, capsaicin treatment and CGRP(8-37) markedly inhibited HCl-induced vasodilation, suggesting that it is neurogenic and mediated by capsaicin sensitive CGRPergic nerves. The finding that capsazepine and CGRP(8-37) inhibited the HCl-induced vasodilation clearly suggest that H^+ activates TRPV1 in the CGRPergic nerves and results in CGRP-mediated vasodilation. Furthermore, perfusion of Krebs solution at low pH levels of 6.0 to 7.2 induced pH-dependent vasodilation as observed in nicotine perfusion. The synergic effects of H^+ and Krebs solution at low pH levels with nicotine imply that protons mediate nicotine-induced vasodilation and act as a transmitter from adrenergic nerves to CGRPergic nerves.

To confirm that protons are a transmitter for mediating nicotine-induced vasodilation, we measured pH levels of the perfusate concomitant with nicotine-induced vasodilation. The results showed that the pH levels decreased as the nicotine-induced vasodilation appeared. Furthermore, a pharmacological study showed that the nicotine-induced pH lowering, as well as the vasodilation, was abolished by cold-storage denervation, guanethidine and mecamylamine. Therefore, it is very likely that nicotine stimulates $\alpha_3\beta_4$ nicotinic ACh receptors to release protons from adrenergic nerves. The released protons activate TRPV1 in the adjacent CGRPergic nerves, releasing CGRP and causing vasodilation. However, capsazepine did not affect the nicotine-induced pH lowering of the perfusate, while the

antagonist inhibited the vasodilation. The contradictory effect of capsazepine may be explained by the hypothesis that capsazepine which has already bound to TRPV1 blocks protons released by nicotine to bind to TRPV1. Therefore, unbound protons in the presence of capsazepine overflow and result in no change, or even a greater decrease in pH levels. Additionally, capsazepine blunts vasodilation mediated by CGRPergic nerves due to blockade of TRPV1 activation by protons.

Nicotine has been shown to stimulate the release of neurotransmitters such as norepinephrine, NPY (Haass *et al*., 1991) and ATP (Von Kugelgen *et al*., 1991) from adrenergic nerve terminals. These results are confirmed by the present finding that nicotine evoked release of norepinephrine, which was abolished by guanethidine. It is widely accepted that the neurotransmitter norepinephrine is stored in adrenergic nerve terminal vesicles and is released into the synapse during signal transduction, spilling norepinephrine and other contents of the vesicle through exocytosis. Norepinephrine in the cytoplasm of the nerve terminal is transported into vesicles using energy provided by the proton gradient, causing protons to be more concentrated inside the vesicle where pH levels are low at 5.5 (Wu *et al*., 2001). This implies that vesicles in adrenergic nerve terminals contain protons to store the transmitter norepinephrine. Therefore, it is likely that protons accompanied by norepinephrine release are released from adrenergic nerves when exocytosis occurs. This notion also suggests that nicotine releases protons from adrenergic nerves, since it evoked norepinephrine release. Furthermore, primary studies showed that

periarterial nerve stimulation of perfused mesenteric arteries resulted in a decrease in pH levels of the perfusate concomitant with initial adrenergic nerve-mediated vasoconstriction followed by CGRPergic nerve-mediated vasodilation, which were abolished by cold-storage denervation. Guanethidine blunted the initial adrenergic vasoconstriction and pH lowering, but it did not affect vasodilation induced by stimulation of CGRPergic nerves. These findings also suggest that protons act as a transmitter for axo-axonal transmission between adrenergic and CGRPergic nerves in mesenteric resistance arteries.

Lee *et al.* (2000) and Si *et al.* (2001) reported that nicotine induces vasodilation in the porcine basilar arteries, which is blocked by guanethidine, α-bungarotoxin and L-NAME, suggesting the hypothesis that nicotine directly acts on presynaptic adrenergic nerve terminals, causing the release of noradrenaline, which then acts on presynaptic β-adrenoceptors located on the NO-containing nerve terminals, resulting in the release of NO and dilation of porcine basilar arteries. These reports are in accordance with the present findings and the hypothesis that nicotine directly stimulates nicotinic acetylcholine receptors on adrenergic nerves to release norepinephrine, which then stimulates TRPV1 on adjacent CGRP-containing nerves to release CGRP and cause vasodilation. Also, these reports and the present findings did not show vasoconstrictor response to nicotine despite the release of norepinephrine. Therefore, it is inferred that coalescence sites between adrenergic nerves and CGRPergic nerves where axo-axonal interaction occurs may locate outside smooth

muscle cells. It is also assumed that nicotinic acetylcholine receptors are only distributed in adrenergic nerves at axo-axonal sites, while adrenergic nerves at neuro-effector (smooth muscle) sites may be not endowed with nicotinic acetylcholine receptors. However, the different distribution of nicotinic acetylcholine receptors in perivascular nerves needs to clarify.

In conclusion, the rat mesenteric resistance arteries are densely innervated by adrenergic vasoconstrictor nerves and NANC CGRPergic vasodilator nerves, which have close contact sites. These nerves have axo-axonal interactions that modulate the vascular nerve function and regulate vascular tone. Our results strongly suggest involvement of protons (H^+) in nicotine-induced vasodilation; we hypothesize, as shown in Figure 10, that nicotine acts on presynaptic nicotinic α3β4 receptors in adrenergic nerves to release protons together with the adrenergic neurotransmitter norepinephrine via exocytosis and then the released protons stimulate TRPV1 receptors on neighboring CGRPergic nerves, resulting in CGRP-mediated vasodilation.

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Footnotes

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Legends for Figures

Figure 1. A typical record (A) showing vasodilation induced by 1-min nicotine perfusion (closed squares) and a line graph (B) showing changes in perfusion pressure (closed diamonds) and overflowed perfusate pH levels (open circles) after perfusion of nicotine (100 μM, indicated by dotted-line ellipse in (A)) in the rat mesenteric vascular bed with active tone and without endothelium. Nicotine (1) and (2) show the 1st series and 2nd series of perfusion, respectively. ACh (closed circle), PNS (diamond) and PPV indicate a bolus injection of acetylcholine (1 nmol), periarterial nerve stimulation (1 Hz) and perfusion of papaverine (100 μM), respectively. SD, perfusion of sodium deoxycholate.

Figure 2. Concentration-dependent vasodilation (A) and decrease in overflowed perfusate pH levels (B) induced by nicotine and their abolishment after cold-storage denervation (C, D) in rat mesenteric vascular beds with active tone and without endothelium, respectively. Nicotine (1) and (2) show the 1st series and 2nd series of perfusion, respectively. *P<0.05, **P<0.01 vs Control.

Figure 3. Effects of guanethidine (A and B), mecamylamine (C and D) and capsazepine (E and F) on nicotine-induced vasodilation and decrease in overflowed perfusate pH levels in rat mesenteric vascular beds with active tone and without endothelium, respectively. *P<0.05, **P<0.01 vs Control.

Figure 4. A typical record (A) showing vasodilation induced by bolus injections of hydrochloric acid (HCl) and a bar graph (B) showing the effects of various agents and treatments on HCl-induced vasodilation in rat

mesenteric vascular beds without endothelium. Each bar indicates means ± SEM in preparations without treatment (Control; n=6) and with treatment of cold-storage denervation (Denervation; n=6), 10 μM capsazepine (Capsazepine; n=4), 0.1 μM CGRP(8-37) (n=4) and 1 μM capsaicin (n=4). ACh, PNS and PPV indicate a bolus injection of acetylcholine (1 nmol), periarterial nerve stimulation (2 Hz) and perfusion of papaverine (100 μM). SD, perfusion of sodium deoxycholate. *P<0.05, **P<0.01 vs Control.

Figure 5. A typical record (A) showing vasodilation induced by 1-min perfusion (closed squares) of Krebs solution at different pH levels of 7.4, 7.2, 7.0, 6.5 and 6.0 and a line graph (B) showing the pH-dependent vasodilation in rat mesenteric vascular beds without endothelium. Each bar indicates means ± SEM of 4 experiments. ACh (closed circle), PNS (inverted triangle) and PPV indicate a bolus injection of acetylcholine (1 nmol), periarterial nerve stimulation (4 Hz) and perfusion of papaverine (100 μM), respectively. SD, perfusion of sodium deoxycholate.

Figure 6. Nicotine-induced release of norepinephrine in perfusate collected every 2 minutes and effect of guanethidine in rat mesenteric vascular beds with active tone and without endothelium. Nicotine (100 μM) was perfused for 1 minute (shown as a closed arrow). ***P*<0.01 vs pre-perfusion values.

Figure 7. Confocal laser photomicrographic images of perivascular nerves in rat mesenteric arteries. Upper images show CGRP (A; green)-like immunoreactive (LI)- and NPY (B; red)-LI-containing fibers. Middle images of C and D which is a magnified image of square area in C are superimposed images of A and B. Lower images indicate CGRP-LI (E; red)

and TRPV1-LI (F; green)-containing fibers. Image G is a superimposed image of E and F. The horizontal bars indicate 100 μm (A, B and C) and 50 μm (D and G).

Figure 8. Scanning electron microscopic images showing perivascular nerves innervating the adventitia of the small mesenteric artery treated with KOH to eliminate collagen fibers. Asterisks and white arrows in A indicate coalescence sites of perivascular nerve fivers and intruding points into vascular smooth muscles, respectively. SM, smooth muscle. Images of B and C show coalescenced nerve fibers (asterisk) and synapse-like contact between two different fibers (a and b), respectively. The horizontal bars indicate 10 μm (A and B) and 5 μm (C).

Figure 9. Immunoscanning electron microscopic images showing CGRP-immunoreactivity (ir)-containing fibers (A) and tyrosine hydroxylase (TH)-ir-containing fibers (B) innervating the small mesenteric artery. Immunoreactivities (ir) were expressed as light red colors. (a), (b) and (c) in images of A and B indicate CGRP-ir-, TH-ir- and non-ir-containing fibers, respectively. An asterisk in B indicates coalescence sites of a fiber (b) with TH-ir and fibers (c) without TH-ir. The horizontal bars indicate 2 μm (A) and 5 μm (B).

Figure 10. Possible mechanisms underlying endothelium-independent vasodilation induced by nicotine in the rat mesenteric artery. Nicotine initially stimulates α3β4 nicotinic receptors on adrenergic nerves. Then, protons together with norepinephrine (NE) are released from adrenergic nerves by exocytosis. Released protons activate TRPV1 (vanilloid)

receptors located on adjacent CGRP nerves to release CGRP and cause vasodilation. N-R indicates nicotinic acetylcholine receptor (α3β4 subtype). NE, α1, CGRP, P2x and NPY show norepinephrine, α1-adrenoceptor, calcitonin gene-related peptide, adenosine triphosphate (ATP)P2x and neuropeptide Y, respectively. R, receptor.

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Table 1. Vascular responses induced by nicotine, dopamine metabolites (3-methoxytyramine, 3,4dihydroxyphenylacetic acid homovanillic acid) and norepinephrine metabolites (normethanephrine, 3,4dihydroxymandelic acid and vanillylmandelic acid) in rat perfused mesenteric vascular beds without endothelium and with active tone.

Agents	N	Vasodilator responses (%)					
		Concentrations (μM)					
		1	3	10	30	100	1000
Nicotine	9	0.7 ± 2.0	3.5 ± 1.8	32.5 ± 4.1	72.6 ± 4.6	82.3 ± 3.1	N/D
Dopamine metabolites							
3-Methoxytyramine	5	4.5 ± 1.4	8.1 ± 2.1	24.0 ± 1.0	49.3 ± 3.8	80.7 ± 2.1	N/D
Capsaicin + 3-Methoxytyramine	5	0.9 ± 1.5	5.9 ± 1.8	24.8 ± 5.3	49.6 ± 5.4	77.4 ± 4.4	N/D
3,4Dihydroxyphenylacetic acid	3	1.6 ± 2.3	-1.6 ± 1.0	-2.4 ± 2.0	-3.1 ± 5.7	-10.0 ± 2.5	N/D
Homovanillic acid	3	-3.1 ± 0.6	-3.3 ± 0.7	-3.8 ± 2.0	-6.1 ± 1.5	-4.9 ± 2.0	N/D
Norepinephrine metabolites							
Normethanephrine	5	0.8 ± 1.3	N/D	13.2 ± 3.4	N/D	47.5 ± 5.1	67.7 ± 5.8
Capsaicin + Normethanephrine	5	-4.1 ± 2.6	N/D	10.1 ± 0.8	N/D	45.6 ± 3.4	74.1 ± 2.8
3,4Dihydroxymandelic acid	3	-0.5 ± 2.4	-1.9 ± 2.4	2.1 ± 8.7	-2.0 ± 5.0	3.7 ± 2.5	N/D
Vanillylmandelic acid	3	1.6 ± 2.4	-8.4 ± 1.8	-6.1 ± 4.4	-6.3 ± 3.3	-13.0 ± 5.3	N/D

N; number of animals, N/D: Not determined

Figure1

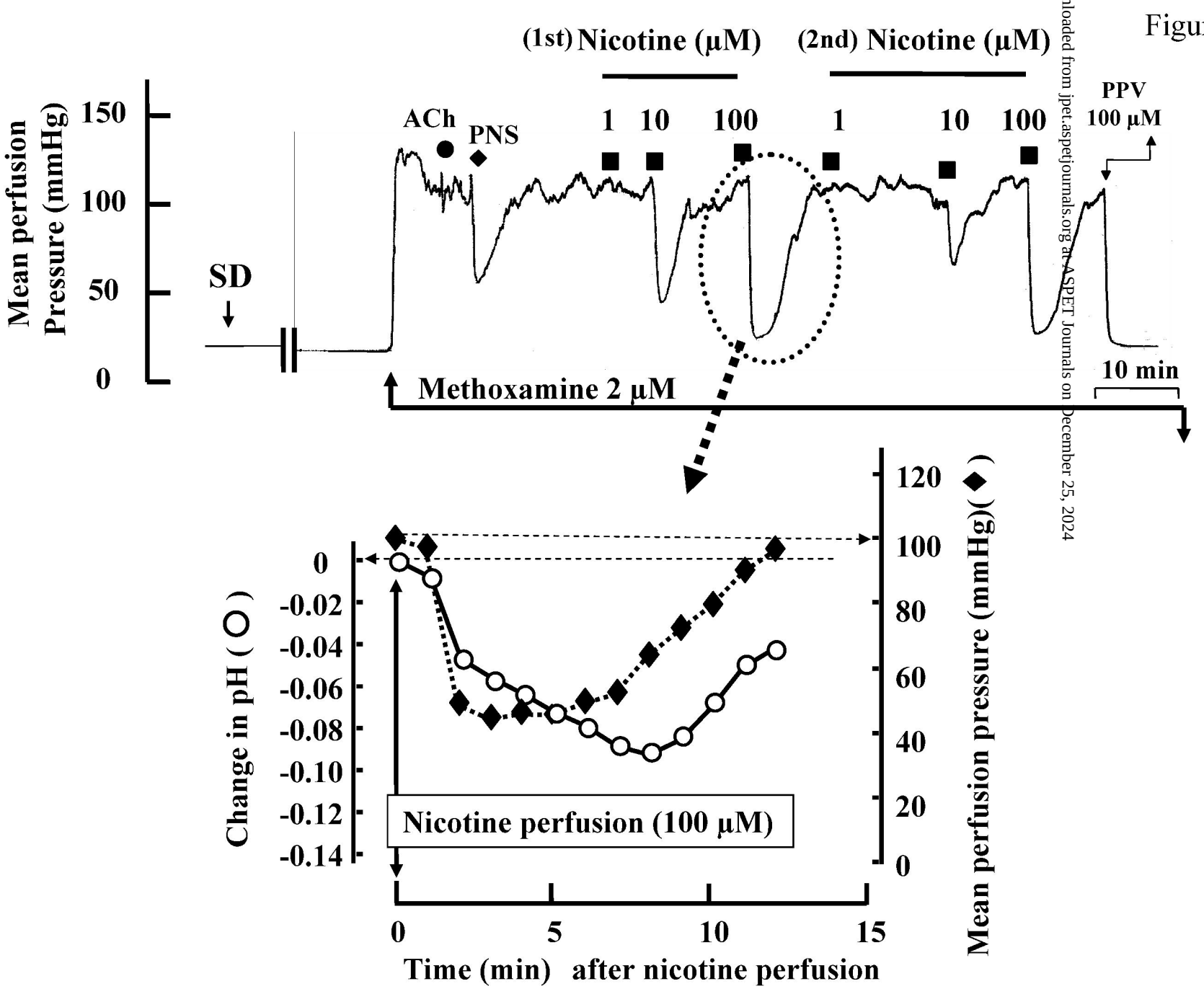
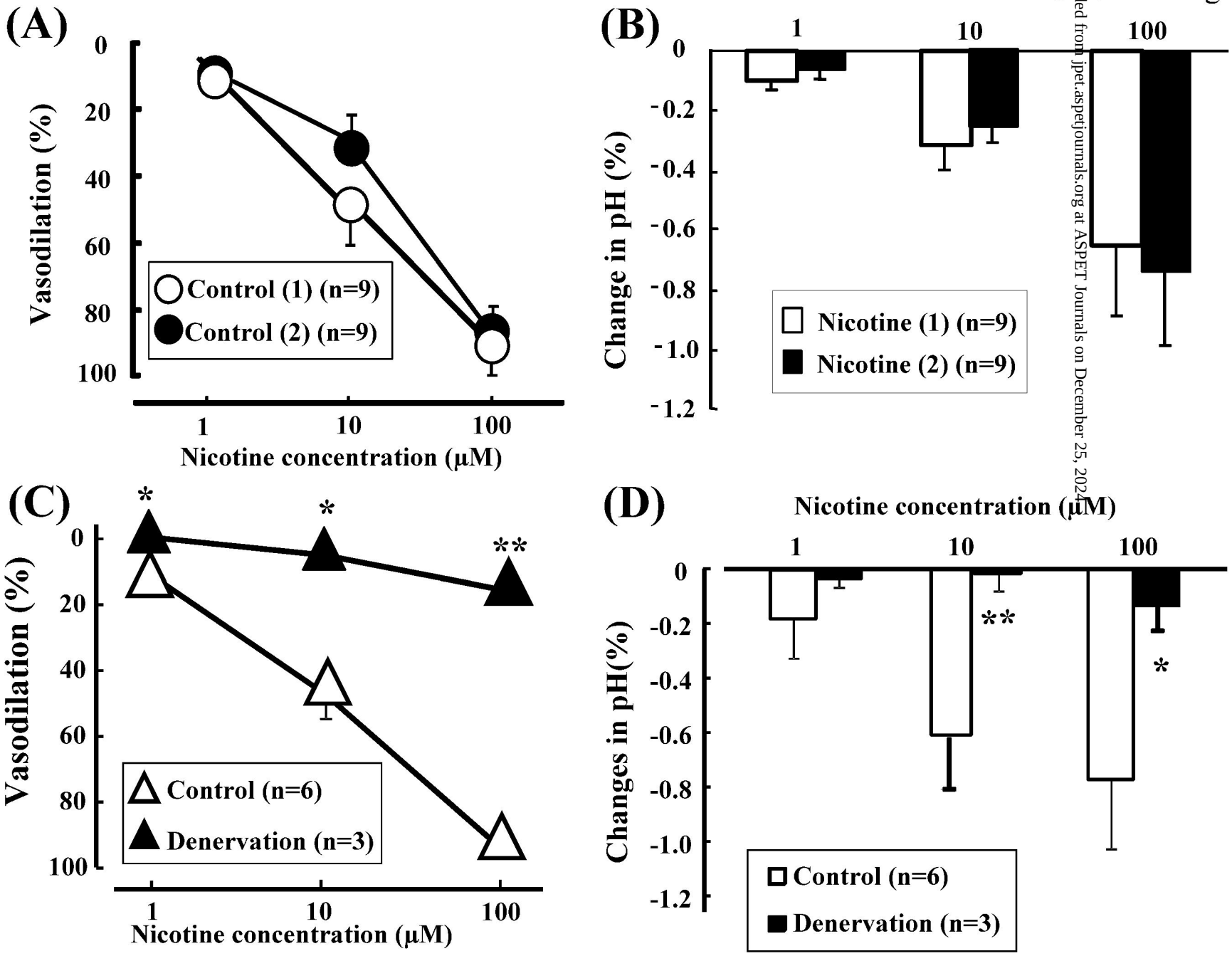


Figure 2



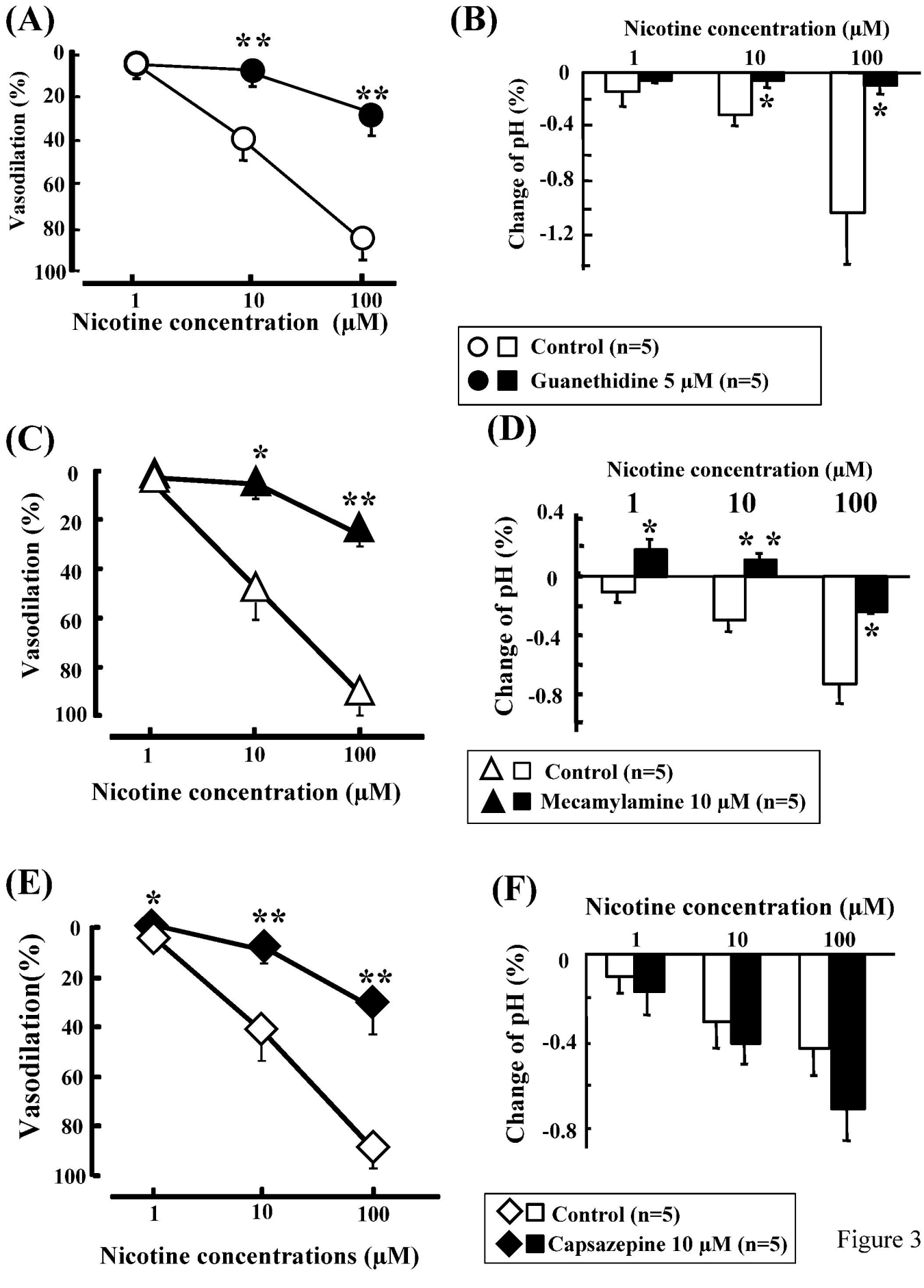

(A)
Vasodilation (%)
0
20
40
60
80
100
**
**
1
10
100
Nicotine concentration (μM)
(B)
Nicotine concentration (μM)
1
10
100
Change of pH (%)
0
-0.4
-0.8
-1.2
*
*
Control (n=5)
Guanethidine 5 μM (n=5)
(C)
Vasodilation (%)
0
20
40
60
80
100
*
**
1
10
100
Nicotine concentration (μM)
(D)
Nicotine concentration (μM)
1
10
100
Change of pH (%)
0.4
0
-0.4
-0.8
*
* *
*
Control (n=5)
Mecamylamine 10 μM (n=5)
(E)
Vasodilation(%)
0
20
40
60
80
100
*
**
**
1
10
100
Nicotine concentrations (μM)
(F)
Nicotine concentration (μM)
1
10
100
Change of pH (%)
0
-0.4
-0.8
Control (n=5)
Capsazepine 10 μM (n=5)

Figure 3

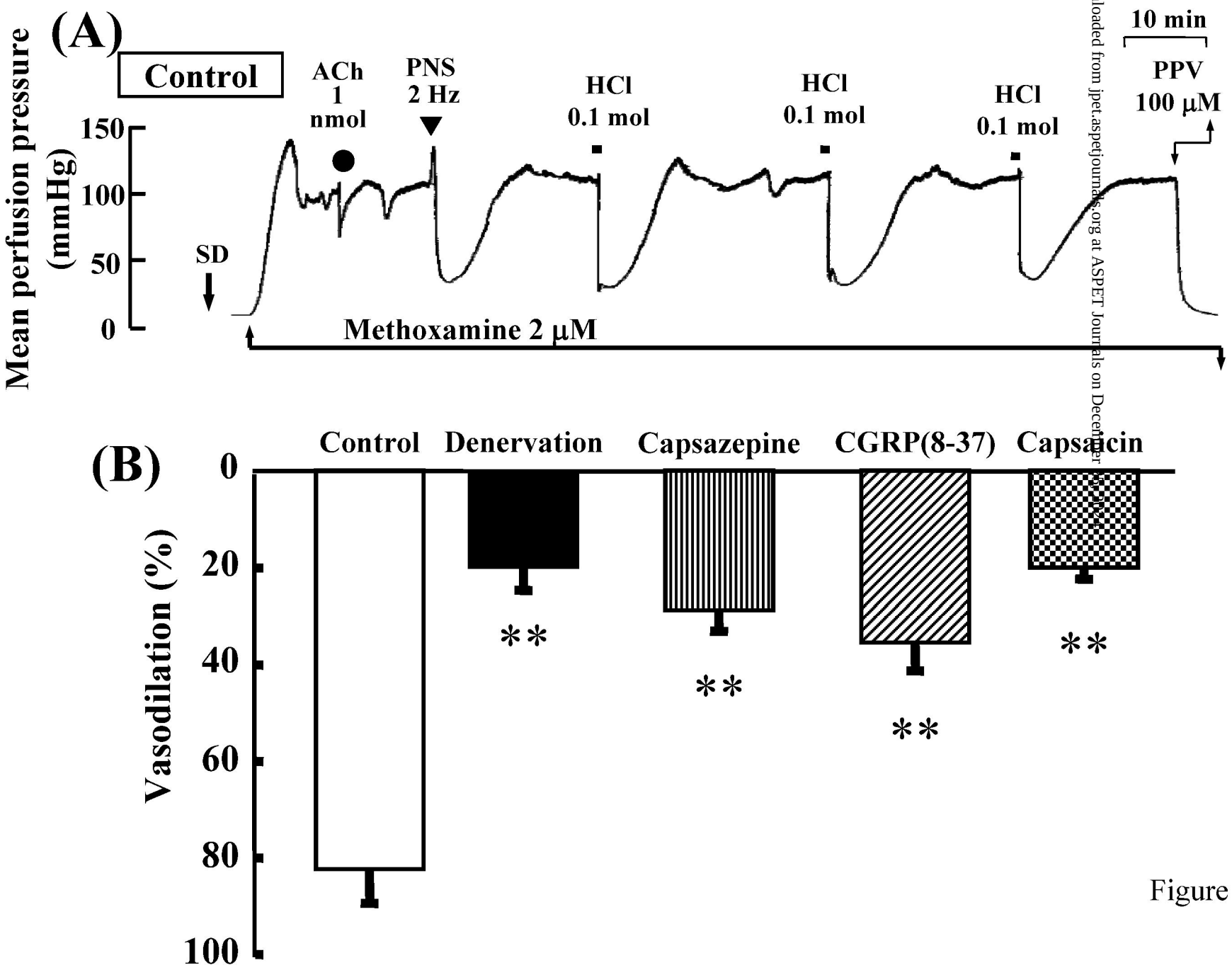


Figure 4

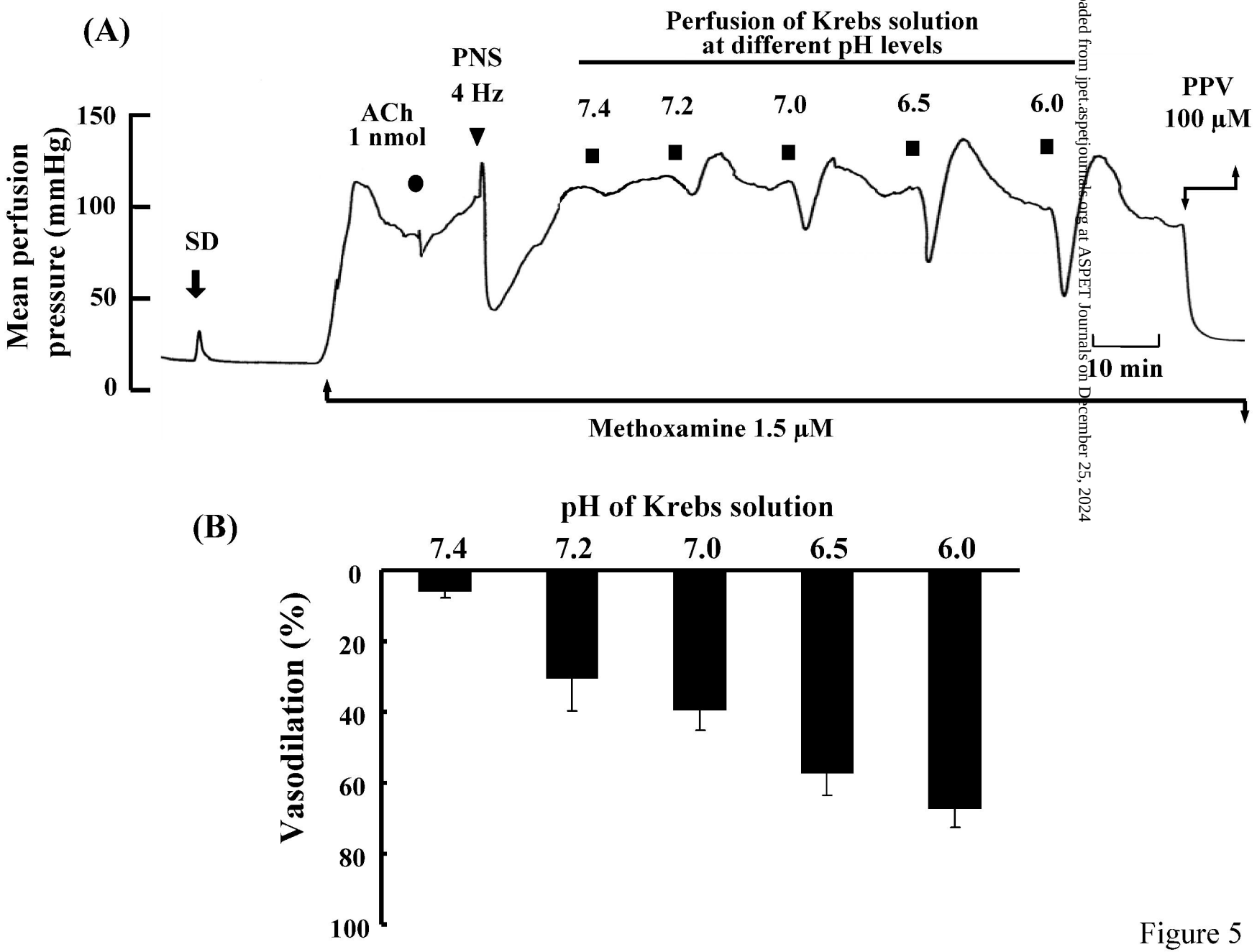
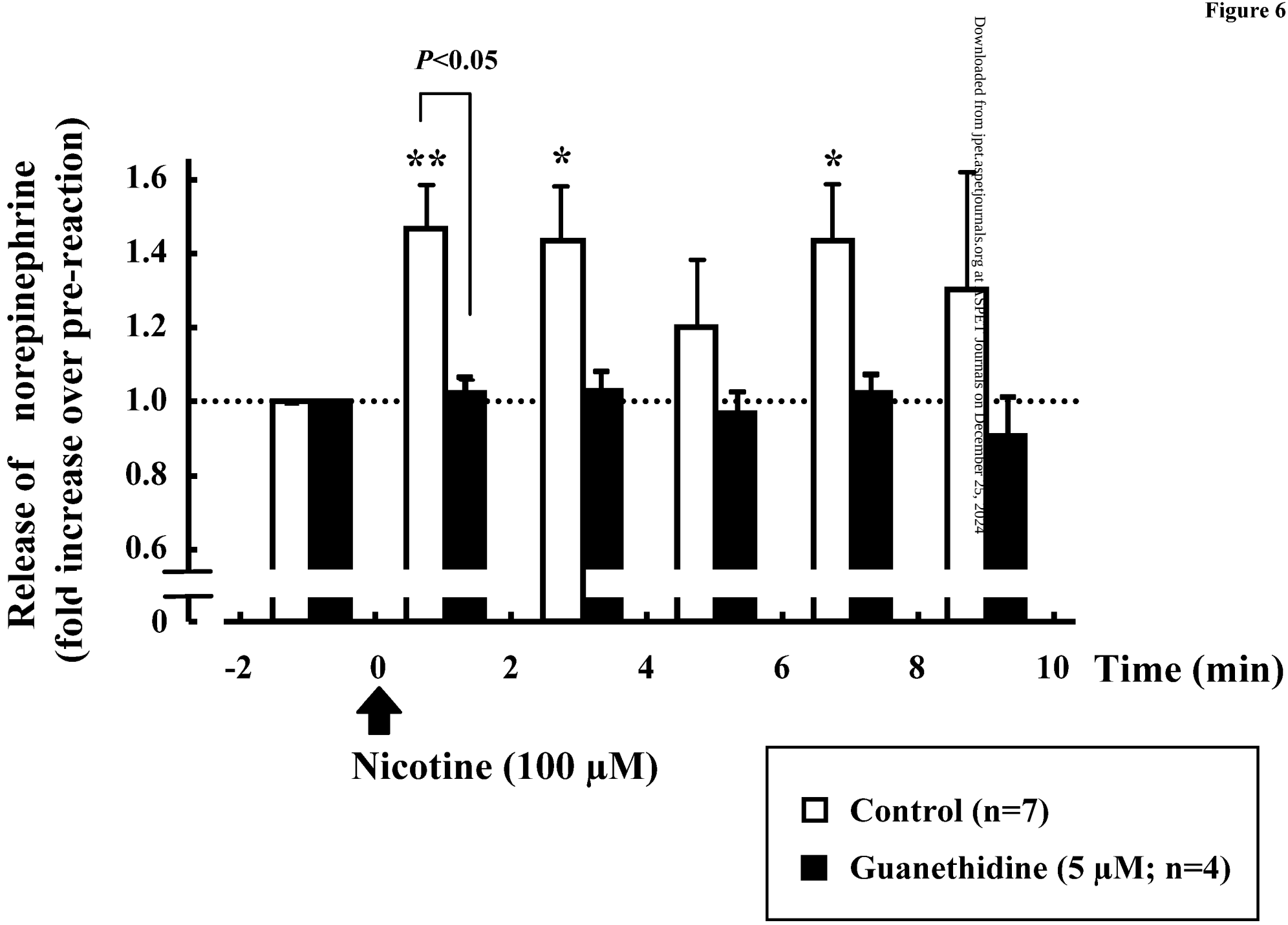


Figure 5

Figure 6



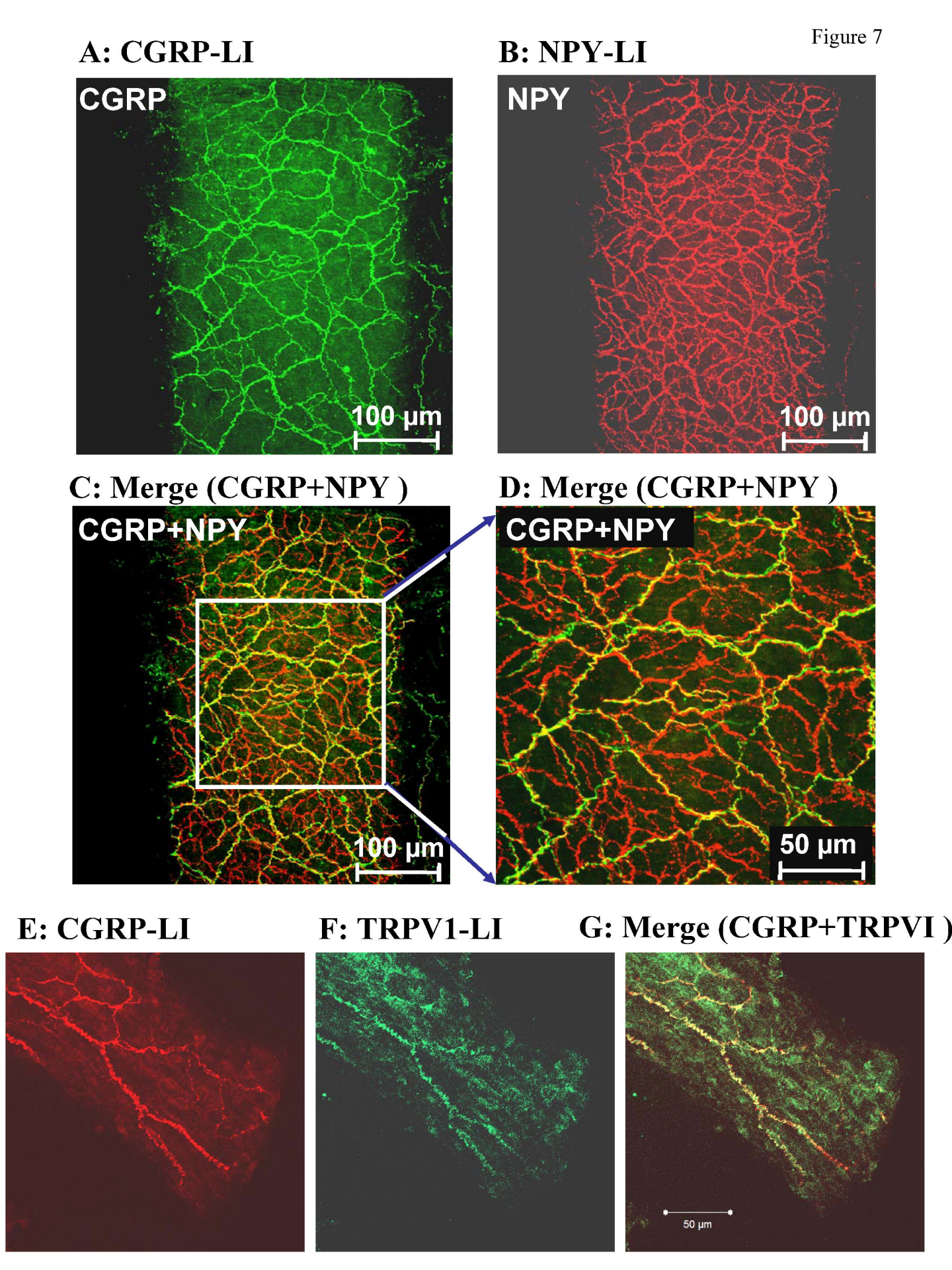

A: CGRP-LI
CGRP
100 µm
B: NPY-LI
NPY
100 µm
C: Merge (CGRP+NPY)
CGRP+NPY
100 µm
D: Merge (CGRP+NPY)
CGRP+NPY
50 µm
E: CGRP-LI
F: TRPV1-LI
G: Merge (CGRP+TRPVI)
50 µm

Figure 8

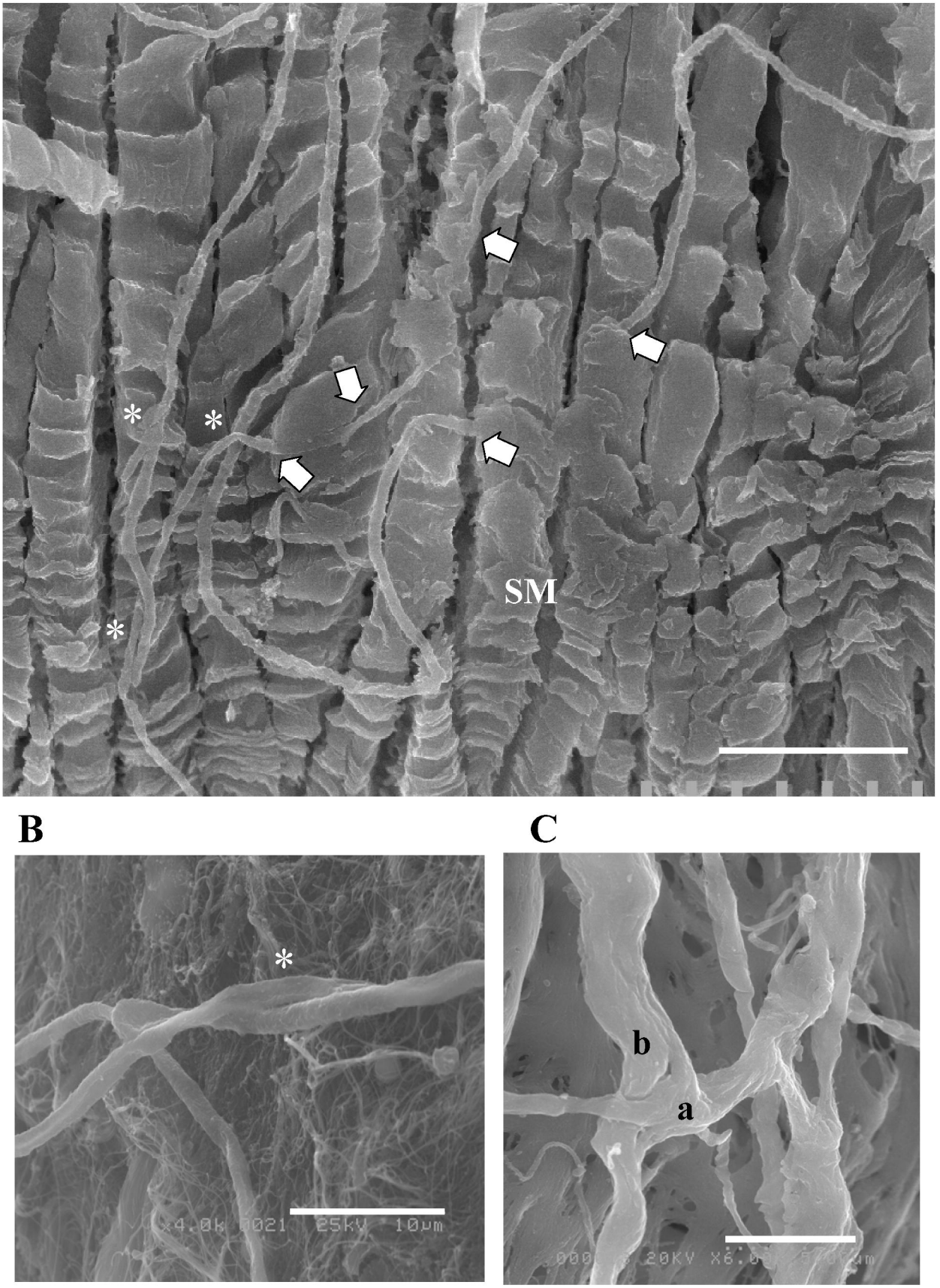


Figure 9

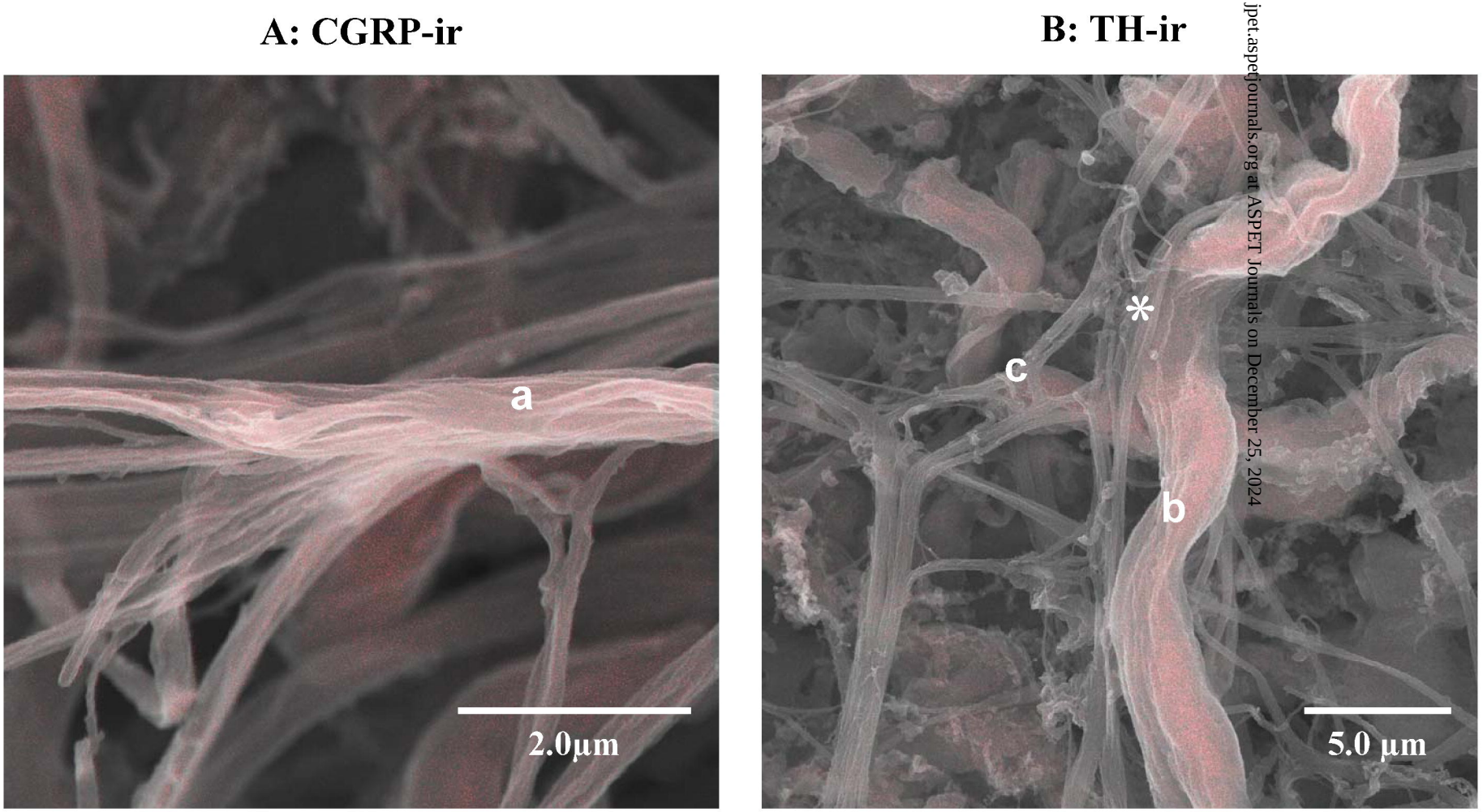


Figure 10