Elsevier Editorial System(tm) for Prostaglandins and Other Lipid Mediators
Manuscript Draft

Manuscript Number: PRO-D-08-00003R1

Title: Characterization of prostanoid receptors present on adrenergic neurons innervating the porcine uterine longitudinal muscle

Article Type: Original Research Article

Section/Category:

Keywords: porcine uterus, prostanoids, prostanoid receptor, adrenergic neurons, noradrenaline release.

Corresponding Author: Dr. Jinshan CAO, PhD DVM

Corresponding Author's Institution: College of Animal Science and Animal Medicine, Inner Mongolia Agriculture University

First Author: Jinshan CAO, PhD DVM

Order of Authors: Jinshan CAO, PhD DVM

Manuscript Region of Origin:

Abstract: The cyclooxygenase-prostanoid pathway regulates myometrial contractility through activation of prostanoid receptors on uterine smooth muscles. However, the possible expression of prostanoid receptors on autonomic nerves can not be excluded completely. The aim of the present study was to clarify the presence of neural prostanoid receptors on adrenergic nerves in the porcine uterine longitudinal muscle. In [3H]-noradrenaline-loaded longitudinal muscle strips of porcine uterus, electrical field stimulation (EFS) evoked [3H]-noradrenaline release in a stimulation frequency-dependent manner. The EFS-evoked release was completely abolished in Ca2+-free (EGTA, 1mM) incubation medium and by tetrodotoxin or ω-conotoxin GVIA, suggesting that [3H]-noradrenaline was released from neural components. The EFS-evoked [3H]-noradrenaline release was significantly enhanced by treatment with indomethacin. In the presence of indomethacin, PGE2 and PGF2α, but not PGD2, inhibited the EFS-evoked [3H]-noradrenaline release. Of synthetic prostanoid receptor agonists examined, both U46619 (TP) and sulprostone (EP1/EP3) decreased

the EFS-evoked [3H]-noradrenaline release in a concentration-dependent manner, while fluprostenol (FP), BW245C (DP) and butaprost (EP2) were almost ineffective. SQ29548 (TP receptor antagonist) blocked the effect of U46619, but SC-19220 (EP1 receptor antagonist) did not change the inhibition by sulprostone or PGE2. Double immunofluorescence staining using protein gene product 9.5, tyrosine hydroxylase, EP3 receptor and TP receptor antibodies suggested the localization of EP3 or TP receptors on adrenergic nerves in the porcine uterus. These results indicated that neural EP3 and TP receptors are present on adrenergic nerves of the porcine uterine longitudinal muscle. Endogenous prostanoid produced by cyclooxygenase can regulate noradrenaline release in an inhibitory manner through activation of these neural prostanoid receptors.

Responses to the comments

Thank you very much for your comments on our manuscript (PRO-D-08-00003).We are pleased to hear that our manuscript is acceptable after a minor revision. We have checked the comments and responded it as follow.

Referee's comments

In the paper, the authors argue that EP3 but not EP1 receptor is an inhibitory neural prostanoid EP receptor located on adrenergic nerves based on the experiments that sulprostone, an EP1/EP3 receptor agonist, inhibits the [3H]-noradrenaline release at a low concentration, and SC-19220 (a EP1 receptor antagonist) had no effect on EFS-evoked [3H]-noradrenaline release. However, it is possible that EP1/EP3 receptors are redundant. Either one of them is enough for the signal transduction. This question can be addressed by examining noradrenaline release using an EP3 antagonist.

Responses

In the porcine longitudinal muscle strips, natural ligand to EP receptor (PGE2) and relative selective ligand to EP1/EP3 receptors (sulprostone) inhibited the EFS-induced 3H-noradrenaline release. Expression of EP1, EP2 and EP3 receptors had been already demonstrated in the porcine uterus. EP2 receptor agonist, butaprost was ineffective to affect noradrenaline release and EP1 receptor antagonist, the concentration of which was 1.5 fold higher than its IC50 values (a new reference, Funk et al., 1993 was added) did not significantly affect the responses to PGE2 and sulprosotone. From these results, EP3 receptor was proposed as an EP receptor subtype mediating the inhibitory actions of both agonists. As the referee mentioned, to confirm the definite involvement of EP3 receptor, it is necessary to investigate the effects of EP3 receptor antagonist on the inhibition by PGE2 and sulprostone. But unfortunately, EP3 receptor is unavailable at commercial base now. In the present experiment, the lack of attenuation by SC19220 did not directly indicate the absence of presynaptic EP1 receptor in the porcine uterine adrenergic fibers. The possibility that the referee mentioned is not excluded completely. Analysis using EP1 selective agonist will be necessary to obtain the clear results. Considering above mentioned points, discussion section has been revised more precisely (see page 16p, Discussion).

Abstract

The cyclooxygenase-prostanoid pathway regulates myometrial contractility through activation of prostanoid receptors on uterine smooth muscles. However, the possible expression of prostanoid receptors on autonomic nerves can not be excluded completely. The aim of the present study was to clarify the presence of neural prostanoid receptors on adrenergic nerves in the porcine uterine longitudinal muscle. In [^{3}H]-noradrenaline-loaded longitudinal muscle strips of porcine uterus, electrical field stimulation (EFS) evoked [^{3}H]-noradrenaline release in a stimulation frequency-dependent manner. The EFS-evoked release was completely abolished in Ca^{2+}-free (EGTA, 1mM) incubation medium and by tetrodotoxin or ω-conotoxin GVIA, suggesting that [^{3}H]-noradrenaline was released from neural components. The EFS-evoked [^{3}H]-noradrenaline release was significantly enhanced by treatment with indomethacin. In the presence of indomethacin, PGE_2 and $PGF_{2\alpha}$, but not PGD_2, inhibited the EFS-evoked [^{3}H]-noradrenaline release. Of synthetic prostanoid receptor agonists examined, both U46619 (TP) and sulprostone (EP_1/EP_3) decreased the EFS-evoked [^{3}H]-noradrenaline release in a concentration-dependent manner, while fluprostenol (FP), BW245C (DP) and butaprost (EP_2) were almost ineffective. SQ29548 (TP receptor antagonist) blocked the effect of U46619, but SC-19220 (EP_1 receptor antagonist) did not change the inhibition by sulprostone or PGE_2. Double immunofluorescence staining using protein gene product 9.5, tyrosine hydroxylase, EP_3 receptor and TP receptor antibodies suggested the localization of EP_3 or TP receptors on adrenergic nerves in the porcine uterus. These results indicated that neural EP_3 and TP receptors are present on adrenergic nerves of the porcine uterine longitudinal muscle. Endogenous prostanoid produced by cyclooxygenase can regulate noradrenaline release in an inhibitory manner through activation of these neural prostanoid receptors.

Key words: porcine uterus, prostanoids, prostanoid receptor, adrenergic neurons, noradrenaline release.

Characterization of prostanoid receptors present on adrenergic neurons innervating the porcine uterine longitudinal muscle

Jinshan Cao[1,2], Tatsuro Nakamura[1], Takio Kitazawa[1], Naoko Yamashiki[3], Tsubasa Yamamoto[3] and Tetsuro Taneike[1]

1) Department of Pharmacology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu Hokkaido 069-8501, Japan

2) Department of Basic Veterinary Medicine, College of Animal Science and Animal Medicine, Inner Mongolia Agriculture University, Hohhot, Inner Mongolia 010018, China

3) Laboratory of Developmental Biology, Faculty of Environmental Systems, Rakuno Gakuen University, Ebetsu Hokkaido 069-8501, Japan

Correspondence: Tetsuro Taneike DVM, PhD.

Department of Pharmacology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu Hokkaido 069-8501, Japan

Tel. 81-11-388-4716,

Fax, 81-11-387-5890

E-mail, taneike@rakuno.ac.jp

Abstract

The cyclooxygenase-prostanoid pathway regulates myometrial contractility through activation of prostanoid receptors on uterine smooth muscles. However, the possible expression of prostanoid receptors on autonomic nerves can not be excluded completely. The aim of the present study was to clarify the presence of neural prostanoid receptors on adrenergic nerves in the porcine uterine longitudinal muscle. In [^{3}H]-noradrenaline-loaded longitudinal muscle strips of porcine uterus, electrical field stimulation (EFS) evoked [^{3}H]-noradrenaline release in a stimulation frequency-dependent manner. The EFS-evoked release was completely abolished in Ca^{2+}-free (EGTA, 1mM) incubation medium and by tetrodotoxin or ω-conotoxin GVIA, suggesting that [^{3}H]-noradrenaline was released from neural components. The EFS-evoked [^{3}H]-noradrenaline release was significantly enhanced by treatment with indomethacin. In the presence of indomethacin, PGE_2 and $PGF_{2\alpha}$, but not PGD_2, inhibited the EFS-evoked [^{3}H]-noradrenaline release. Of synthetic prostanoid receptor agonists examined, both U46619 (TP) and sulprostone (EP_1/EP_3) decreased the EFS-evoked [^{3}H]-noradrenaline release in a concentration-dependent manner, while fluprostenol (FP), BW245C (DP) and butaprost (EP_2) were almost ineffective. SQ29548 (TP receptor antagonist) blocked the effect of U46619, but SC-19220 (EP_1 receptor antagonist) did not change the inhibition by sulprostone or PGE_2. Double immunofluorescence staining using protein gene product 9.5, tyrosine hydroxylase, EP_3 receptor and TP receptor antibodies suggested the localization of EP_3 or TP receptors on adrenergic nerves in the porcine uterus. These results indicated that neural EP_3 and TP receptors are present on adrenergic nerves of the porcine uterine longitudinal muscle.

Endogenous prostanoid produced by cyclooxygenase can regulate noradrenaline release in an inhibitory manner through activation of these neural prostanoid receptors.

Key words: porcine uterus, prostanoids, prostanoid receptor, adrenergic neurons, noradrenaline release.

1. Introduction

Prostanoids, a group of lipid mediators including prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2) and thromboxane A_2 (TXA_2), are synthesized from arachidonic acid by cyclooxygenase and specific prostanoid synthases. The synthesized prostanoids are immediately secreted into extracellular fluid via a prostanoid-specific transporter expressed in the cytoplasmic membrane and act on specific prostanoid receptors (FP, EP_1, EP_2, EP_3, EP_4, DP, IP, TP) in an autocrine or paracrine manner [1, 2]. Prostanoids play important roles in generation of heat, modulation of pain, induction of sleep, aggregation of platelets, protection of gastric mucosa and modulation of smooth muscle tonus. Since the first discovery of prostanoids in semen and seminal glands, their physiological function in reproductive organs has attracted a great deal of attention, and several studies have demonstrated that the cyclooxygenase, synthetized prostanoids and coupled prostanoid receptors play essential physiological roles in induction of parturition via modulation of uterine smooth muscle contractile activity and via a hormonal action for luteolysis [3-9]. Prostanoid receptors expressed in the uterus have been investigated in functional and molecular biological studies, and these studies have indicated a species-related heterogeneous population of prostanoid receptors in the uterus of various mammals except for the pig [10]. Therefore, we have analyzed prostanoid receptor subtypes in the porcine uterus and have shown a muscle layer-dependent distribution of prostanoid receptors (EP_1, EP_2, EP_3, FP, longitudinal muscle > circular muscle; DP, IP, TP, circular muscle >longitudinal muscle). The prostanoid receptor subtypes present in the porcine uterus are consistent with those demonstrated in the human uterus. Cyclooxygenase-prostanoid-prostanoid receptor

mechanisms were found to be involved in the regulation of spontaneous myometrial activity in the non-pregnant porcine uterus [11-14].

Besides hormonal regulatory factors, such as prostanoids and sex steroids, autonomic nerves are also thought to be important for regulation of coordinated uterine contractility. Functional studies by electrical field and extrinsic nerve stimulation have provided evidence of excitatory cholinergic (rat, human and pig) as well as α-excitatory and/or β-inhibitory adrenergic innervation of the uterine body and cervix (rat, human, rabbit, guinea-pig and pig) [15-22]. Autonomic nerves, exclusively adrenergic fibers, have been shown to eliminate during pregnancy and to regenerate in postpartum in the uterus [23-27], suggesting a significant contribution of uterine autonomic nerves to quiescence of the uterus during pregnancy. Since presynaptic nerve terminals possess release-modulating autoreceptors and heteroreceptors, it is likely that some bioactive substances can affect neuro-effector transmission in the uterus and regulate myometrial contractility indirectly.

Although mechanical responses of prostanoids in isolated visceral smooth muscle preparations such as the uterus, gastrointestinal tract, blood vessels and trachea were evoked by direct action through smooth muscle prostanoid receptors [10-12, 28-32], some studies have revealed the presence of presynaptic prostanoid receptors modulating the electrical stimulation-evoked release of noradrenaline from adrenergic neurons in rabbit mesenteric arteries, human iris-ciliary, bovine irides, mouse cultured sympathetic neurons, rat mesenteric bed and stomach [33-39]. Therefore, also in the uterus, prostanoids might affect myometrial contraction through the regulation of neurotransmitter release through presynaptic receptors.

The aim of the present study was to clarify whether neuronal prostanoid receptors are present on adrenergic neurons of porcine uterine longitudinal muscles. To accomplish this objective, the effects of naturally occurring prostaglandins and selective prostanoid receptor agonists on electrical field stimulation (EFS)-evoked [^{3}H] efflux were examined in [^{3}H]-noradrenaline-loaded muscle preparations. Distribution of prostanoid receptors on neural elements was visualized by double immunofluorescence staining using protein gene product 9.5 (maker of neurons), tyrosine hydroxylase (marker of adrenergic neurons) and prostanoid receptor antibodies (TP and EP_3 receptors).

2. Methods

2.1. Tissue preparation

Fresh uteri, with the ovaries intact, from 60 sexually mature crossbred virgin gilts (about 6 months old, non-pregnant) were provided by a local abattoir and used in the experiments on the day of slaughter. The pigs were judged to be in proestrus by gross examination of follicle size (smaller than 2 mm in diameter) and by appearance of corpora lutea [40]. After cutting off the endometrium, myometrial strips parallel to the direction of the longitudinal muscle fibers were isolated as described previously [11, 41] and used in the following experiments. Exclusive adrenergic innervation of porcine uterine longitudinal muscle has been already demonstrated by contractions studies [20, 22]

2.2. [^{3}H]-Noradrenaline release study

The isolated longitudinal muscle preparations were incubated with 100 nM

[^{3}H]-noradrenaline for 40 min in 2.8 ml warmed incubation medium (37°C, 118.6 mM NaCl; 4.7 mM KCl; 2.52 mM $CaCl_2$; 1.23 mM $MgSO_4$; 25 mM $NaHCO_3$; 10 mM glucose; 0.3 mM ascorbic acid; 0.031 mM disodium EDTA) [42], equilibrated with 95% O_2 + 5% CO_2. After loading [^{3}H]-noradrenaline, the muscle strips were washed three times (for 10 min each time) in 5 ml fresh incubation medium containing 400 nM desipramine (inhibitor of neuronal noradrenaline uptake) and 1 μM yohimbine (antagonist of presynaptic α_2-adrenoceptor). Then the washed muscle strips were immersed in 5 ml incubation solution (bubbled with 95% O_2+5% CO_2 mixture, 37°C). The incubation medium was sequentially changed at 5 min intervals. In order to stimulate adrenergic nerves, EFS (2, 5, 10, 20 Hz, 50 V, 0.5 ms in duration, for 30 s) was applied through two platinum ring electrodes fixed on the top and bottom of the muscle strips. At the end of each experiment, the tissues were dissolved in 500 μl Soluene and the radioactivity was measured together with each fraction in a liquid scintillation counter. The [^{3}H]-noradrenaline outflow was expressed as the fractional rate, in which the amount of radioactivity in the incubation solution was divided by the total radioactivity present in the tissue, at the same collection period. The [^{3}H]-noradrenaline content of the tissue in each period was calculated by adding cumulatively the amount of [^{3}H]-noradrenaline in each fraction to the [^{3}H]-noradrenaline content of the tissue at the end of the experiments. Most release studies were performed using incubation medium containing indomethacin (3 μM) to exclude the influence of endogenous prostanoid. However, some experiments were carried out in the absence of indomethacin and the influence of endogenous prostanoid on [^{3}H]-noradrenaline was estimated.

Effects of tetrodotoxin, ω-conotoxin and prostanoid receptor agonists on the EFS-induced [^{3}H]-noradrenaline release were examined as follows. First, EFS (10 Hz, 50 V, 0.5 ms in duration, for 30s) was applied to [^{3}H]-noradrenaline-loaded muscle strips at 20 min (S1) of the series of experiments, and a second stimulation (S2) was applied 30 min later of S1 in the absence (control) or presence of drugs. Inhibitory or excitatory effects on the EFS-evoked release were evaluated by comparison of the fractional rate value of S2 or S2/S1 ratio. When the effects of prostanoid receptor antagonists were examined, the antagonists were added to the incubation medium 30 min before application of prostanoids.

2.3. Immunofluorescence confocal laser microscopy

Uterine strips (10 mm long) obtained from the cornu were fixed immediately in 4% para-formaldehyde-PBS (pH 7.2) overnight at 4°C. The fixed uterine samples were transferred to 0.1 M PBS containing 30% sucrose and dehydrated over a period of 36 h at 4°C. The uterine tissues were then embedded in O.C.T compound, snap-frozen in liquid nitrogen, and stored at -80 °C until use. All tissue blocks were cut into 10-12-μm-thick sections using a Frigocut cryostat (LEICA, CM 1500). The sections were put on MAS-coated microscope slides and air-dried. After the sections had been washed three times with 0.01 M PBS solution for 10 min each time, the cytoplasmic membranes were permeated with 3% triton X-100-PBS solution (for 30 min) and then washed again three times with 0.01 M PBS solution for 10 min each time. Normal goat serum (100 μl) was put on each section, and the sections were incubated for 1 h. After removing the superfluous goat serum, a mixture of tyrosine hydroxylase (TH; Novus

Biologicals Inc, Littleton, USA; 1:500) or protein gene product 9.5 (PGP 9.5; Abcam, Tokyo, Japan; 1:500) antibodies and EP_3 receptor (Santa Cruz Biotechnology, Inc, California, USA; 1:400) or TP receptor (Santa Cruz Biotechnology, Inc, California, USA; 1:400) antibodies was applied, and the sections were incubated overnight in a humidified chamber at room temperature. After the sections had been washed in 0.01 M PBS 5 times for 10 min each time, a mixture of FITC-conjugated goat-anti-mouse secondary antibody (for detection of TH and PGP9.5; 1:100; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) and rhodamine-conjugated goat-anti-rabbit secondary antibody (for detection of EP_3 and TP receptors; 1:500; ICN Pharmaceuticals, Inc., Ohio, USA) was applied, and the sections were reacted for 2 h at room temperature. After washing, each section was mounted using vectashield mounting medium for fluorescence (Vector Laboratories, Inc., CA, USA). Microphotographs were taken using an Olympus Fluoview confocal laser-scanning microscope (Olympus, Tokyo, Japan). Normal goat serum did not give positive immunohistochemical reactions in any case.

2.4. Chemicals

Atropine sulfate, desipramine hydrochloride, indomethacin, phentolamine hydrochloride and yohimbine hydrochloride were obtained from Sigma-Aldrich (Tokyo, Japan). Butaprost (9-oxo-11α,16R-dihydroxy-17-cyclobutyl- prost-13E-en-1-oic acid, methyl ester), BW245C ((4S)-3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid), fluprostenol ((±)-9α,11α,15R-trihydroxy-16-(3-(trifluoromethyl)phenoxy)-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-oic acid), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2),

SC19220 (8-chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl) hydrazide), SQ29548 (1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid), sulprostone (N-(methylsulfonyl)-9-oxo-11α,15R-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide) and U46619 (7-[6-(3-hydroxy-1-ocetenyl)-2-oxabicyclo[2.2.1]hept-5yl]-,[1R-[1α,4α, 5β(Z),6α(1E,3S*)]]-5-heptenoic acid) were obtained from Cayman Chemical Company (MI, USA). Propranolol, tetrodotoxin and ω-conotoxin GVIA were obtained from Tocris Bioscience (MO, USA). Butaprost, indomethacin, fluprostenol, PGE_2, $PGF_{2\alpha}$, PGD_2, sulprostone and U46619 were dissolved in ethanol. SC19220, SQ29548 and BW245C were dissolved in dimethylsulfoxide. Atropine, desipramine, phentolamine, propranolol, yohimbine, tetrodotoxin and ω-conotoxin GVIA were dissolved in distilled water. The maximum concentrations of dimethylsulfoxide and ethanol in the bathing solution were set below 0.05%. These vehicles did not change EFS-evoked release of [^{3}H]-noradrenaline from longitudinal muscle strips of the non-pregnant porcine uterus.

2.5. Statistics

Results of the experiments are expressed as means ± S.E.M. of uterine smooth muscle preparations obtained from at least four different pigs. Unpaired Student's t-test was used for statistical comparison between two groups. A *P* value of 0.05 or less was considered statistically significant.

3. Results

3.1. EFS-induced [^{3}H]-noradrenaline release in the longitudinal muscle

EFS increased [^{3}H]-noradrenaline release from longitudinal muscle strips in a frequency-dependent manner (2-20 Hz). EFS-induced noradrenaline release was compared both in the absence and presence of indomethacin (3 μM), a nonselective inhibitor of cyclooxygenase. Although the fractional rates of spontaneous noradrenaline release at first fraction were almost the same regardless of the presence (0.75 ± 0.05 % 5 min^{-1}, n=6) or absence of indomethacin (0.83 ± 0.07 % 5 min^{-1}, n=6), EFS-induced increase of fractional rates in the presence of indomethacin (2Hz = 1.15 ± 0.09 % 5 min^{-1}, 5Hz = 1.76 ± 0.21 % 5 min^{-1}, 10Hz = 2.84 ± 0.32 % 5 min^{-1}, 20 Hz = 3.29 ± 0.32 % 5 min^{-1}, n=9) were significantly higher than those in the absence of indomethacin (2Hz = 0.94 ± 0.05 % 5 min^{-1}, 5Hz = 1.16 ± 0.15 % 5 min^{-1}, 10Hz = 1.67 ± 0.22 % 5 min^{-1}, 20 Hz = 2.16 ± 0.24 % 5 min^{-1}, n=6) (Fig. 1). Modulation of EFS-induced noradrenaline release by endogenous prostanoid was further evaluated using S2/S1 ratio of 10-Hz stimulation. The S2/S1 ratio was 0.64 ± 0.07 (n=8) in the absence of indomethacin. On the other hand, after observing S1 in the absence of indomethacin, the preparations were treated with indomethacin for 30 min and then S2 was applied. The obtained S2/S1 ratio (0.89 ± 0.06, n=8) was significantly higher than that in the absence of indomethacin (P<0.05). Therefore, in the subsequent experiments, to examine the effects of bath-applied prostanoid, [^{3}H]-noradrenaline release study was performed in the presence of indomethacin (3 μM).

To characterize noradrenaline release evoked by EFS, effects of tetrodotoxin (1 μM; Fig. 2B) and ω-conotoxin GVIA (1 μM; Fig. 2C) were examined. As indicated in Fig. 2A, EFS (10 Hz) caused a reproducible [^{3}H]-noradrenaline release at 30-min intervals (fractional rate: S1=2.93 ± 0.46% 5 min^{-1}, S2=2.82 ± 0.41 % 5 min^{-1}, P=0.85,

n=8). However, pretreatment with tetrodotoxin (1 μM) or ω-conotoxin GVIA (1 μM) almost completely abolished the second EFS (S2)-induced [^{3}H]-noradrenaline release. In addition, EFS-induced [^{3}H]-noradrenaline release was also abolished in Ca^{2+}-free incubation medium containing 1 mM EGTA (Fig. 2D).

3.2. Effects of prostanoids on EFS-induced [^{3}H]-noradrenaline release

First, effects of three naturally occurring prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGD_2) on EFS-induced [^{3}H]-noradrenaline release were examined to evaluate the presynaptic action of prostaglandins. Concentration of each prostaglandin was fixed at 1 μM, enough concentration to act on prostanoid receptors. PGE_2, $PGF_{2\alpha}$ or PGD_2 (1 μM) itself did not affect the spontaneous [^{3}H]-noradrenaline release in the present experimental conditions (data not shown). However, these prostaglandins had various effects on EFS-evoked [^{3}H]-outflow. As shown in Fig. 3, PGE_2 and $PGF_{2\alpha}$ significantly decreased the EFS-evoked [^{3}H]-noradrenaline release from uterine strips, but PGD_2 did not show any significant effects.

Since naturally occurring prostaglandins can cross act several prostanoid receptor subtypes [43], some selective synthetic prostanoid receptor agonists were tested for further clarification of prostanoid receptor subtypes inhibiting noradrenaline release. Sulprostone, an EP_1/EP_3 receptor agonist, inhibited EFS-evoked [^{3}H]-noradrenaline release in a concentration-dependent manner (1 nM-1 μM) (Fig. 4A), but the EP_2 receptor agonist butaprost did not change the [^{3}H]-outflow even at 1 μM (Fig. 4B). The EP_1 receptor antagonist SC-19220 (10 μM) did not affect the inhibitory effect of PGE_2 (Fig. 4B). The S2/S1 values were 0.46 ± 0.06 (n=5) in the absence and 0.59 ± 0.11

(n=4) in the presence of SC-19220, respectively (P=0.25). Sulprostone (100 nM)-induced inhibition (S2/S1=0.61 ± 0.04, n=7) was not also significantly affected by SC-19220 (10 μM) (S2/S1=0.54 ± 0.08, n=5, P=0.3) (Fig. 4B). In contrast to the significant inhibitory effect of $PGF_{2\alpha}$, fluprostenol (1 μM), an FP receptor agonist, did not have an inhibitory effect on EFS-induced [^{3}H]-noradrenaline release (Fig. 5). On the other hand, similar to PGD_2, a natural DP receptor agonist, BW245C (1 μM), a synthetic DP receptor agonist, did not affect the EFS-induced [^{3}H]-noradrenaline release (S2/S1= 0.99 ± 0.18, n=6).

The effect of the TP receptor agonist U46619 on EFS-evoked [^{3}H]-noradrenaline release was also examined. U46619 was found to be a potent inhibitor of the EFS-induced [^{3}H]-noradrenaline release. Fig. 6A shows the concentration-dependent (10 nM-10 μM) inhibition of noradrenaline release by U46619, and these inhibitory responses were effectively blocked by SQ29548, a TP receptor antagonist. However, SQ29548 itself did not change the EFS-evoked [^{3}H]-noradrenaline release (Fig. 6B).

3.4. Localization of EP_3 and TP receptors on adrenergic neurons

In order to investigate the localization of EP_3 and TP prostanoid receptors on adrenergic nerves in the porcine myometrium, we performed double-immunofluorescence staining using PGP 9.5 antibody (a pan-neuronal marker), TH antibody (an adrenergic marker), and EP_3 receptor and TP receptor antibodies. As previously shown by functional and immunohistochemical studies [11, 14, 20, 21], EP_3 receptor was mainly localized on smooth muscle elements of the porcine uterus (Fig. 7A and D), and positive nerve fibers for PGP 9.5 and TH antibodies (adrenergic nerves)

were distributed among smooth muscle cells (Fig. 7C and E). However, mergence of EP_3-positive elements and adrenergic fibers (yellow color, Fig. 7C and F) strongly suggested the localization of EP_3 receptor on adrenergic neurons in the porcine uterus. Similar to the case of EP_3 receptors, the merged photographs clearly indicated the expression of TP receptors on adrenergic neurons of porcine uterine longitudinal muscle layers (Fig. 8).

4. Discussion

In the present study, we first demonstrated that inhibitory EP_3 and TP receptors are present on adrenergic neurons of the porcine uterus and that endogenous prostanoid has an inhibitory effect on neuro-effector transmission through these neural receptors. This is an unique additional mechanism for functional roles of prostanoid-prostanoid receptor pathways in the female reproductive organs. Prostanoid might regulate the myometrial contractility through well-known direct action on smooth muscle cells and indirect action by modulation of neuro-effector transmission.

Adrenergic neurons are major autonomic innervation in the mammalian myometrium and regulate uterine contractility [23-27, 44, 45]. In the porcine uterine longitudinal muscle strips treated with atropine and propranolol, EFS evoked myometrial contractions which were blocked by phentolamine [20, 21]. These results clearly indicate the functional innervation of adrenergic nerves in the porcine uterine longitudinal muscle. Adrenergic innervation in the uterus was also confirmed by the results of present immunohistochemical analysis showing the presence of TH-immunopositive fibers because TH is a rate-limiting enzyme in synthesis of

noradrenaline. Modulation of adrenergic neuro-effector transmission by prostanoid receptor agonists was examined in this longitudinal muscle preparation with focus on the release of noradrenaline from adrenergic nerves.

EFS applied to myometrium strips preloaded with [^{3}H]-noradrenaline caused a frequency-dependent release of [^{3}H]-noradrenaline, and stimulation frequency-release relationships were similar to stimulation frequency-contraction relationships reported previously [21]. Tetrodotoxin, a neuronal Na^+ channel blocker, and ω-conotoxin GVIA, a neuronal voltage-activated Ca^{2+} channel blocker, inhibited the EFS-induced release of autonomic neurotransmitters in the guinea-pig artery [46] and rat mesenteric artery [47]. In our study, EFS-evoked [^{3}H]-noradrenaline release was completely abolished by tetrodotoxin and ω-conotoxin GVIA and in Ca^{2+}-free (EGTA) solution. These results indicated that [^{3}H]-noradrenaline was released from neural components of the porcine uterine longitudinal muscles by EFS.

To evaluate the possible contribution of endogenous prostanoid to noradrenaline release, stimulation frequency-release relationships were established both in the absence and presence of indomethacin, and then EFS-induced noradrenaline release was compared. Although the basal spontaneous release was almost the same, EFS-evoked [^{3}H]-noradrenaline release at every frequency in the indomethacin-treated muscle strips was higher than that in the absence of indomethacin. The S2/S1 ratio of 10 Hz-induced release increased significantly in the presence of indomethacin (absence vs. presence: 0.64 vs. 0.89). These results indicate an inhibitory modulation of noradrenaline release from adrenergic nerves by endogenous prostanoid. In the present experiment, to clarify the effect of exogenous prostanoids, synthesis of endogenous prostanoid was blocked by

indomethacin as previously reported in guinea-pig trachea and rat [39, 48].

EP_3 receptor-mediated inhibition of EFS-evoked [^{3}H]-noradrenaline release by PGE_2 and sulprostone has been already demonstrated in the rabbit aorta [34], mouse cultured sympathetic neurons [36] and rat mesenteric bed [38]. Similar inhibition of noradrenaline release by PGE_2 and sulprostone was observed in the porcine uterus. PGE_2 is an endogenous ligand for EP receptors (EP_1, EP_2, EP_3 and EP_4), and the presence of EP_1, EP_2 and EP_3 receptors was demonstrated in the porcine uterus pharmacologically [11]. In the present study, butaprost (EP_2 receptor agonist) had no effect on EFS-evoked [^{3}H]-noradrenaline release and the EP_1 receptor antagonist SC-19220 (10 μM, IC_{50} for EP_1 was 6.7μM [49]) did not change the PGE_2-induced inhibition. Sulprostone, an EP_1/EP_3 receptor agonist, inhibited the [^{3}H]-noradrenaline release from a low concentration (from 1 nM, Ki for EP_1= 21 nM, Ki for EP_3=0.6 nM, [50]) and SC19220 did not change the inhibition by sulprostone. From these results, EP_3 receptor was most probable as a presynaptic prostanoid EP receptor involved in the PGE_2- and sulprostone-induced inhibition. However pharmacological analysis using EP_1 receptor agonist (effects on EFS-induced noradrenaline release) and EP_3 receptor antagonist (effects on the inhibitory responses to sulprostone) might be needed for further characterization of EP receptor subtype. Confocal images of double immunofluorescence staining of nerve fibers and EP_3 receptors in the porcine uterus clearly suggest the localization of EP_3 receptors on adrenergic neural elements and these immunohistochemical evidences support the results of the present noradrenaline release study.

Although $PGF_{2\alpha}$ decreased the EFS-induced [^{3}H]-noradrenaline release, fluprostenol

(FP receptor selective agonist) did not change the evoked release. Since affinities of $PGF_{2\alpha}$ (Ki=3 nM) and fluprostenol (Ki=4 nM) for FP receptor were almost equipotent [50], the discrepant actions between both agonists suggest that activation of FP receptor had no effect on noradrenaline release and that inhibition by $PGF_{2\alpha}$ is probably caused by activation of other prostanoid receptors. The Ki value of $PGF_{2\alpha}$ for EP_3 receptor (75 nM) was only 22-times higher than that for FP receptor (Ki=3.4 nM) [50]. Therefore, it might be likely that $PGF_{2\alpha}$ acts on EP_3 receptor to inhibit noradrenaline release at 1 μM. However, further study using EP_3 receptor antagonist would be needed. Although the presence of DP receptors in the uterus has only been demonstrated in humans [10] and pigs [11, 12], neither PGD_2 nor BW245C (DP receptor agonist) decreased the EFS-evoked [^{3}H]-noradrenaline release. Since the expression of IP receptors in longitudinal muscle layer of the porcine uterus was low [11] and since there has been no previous report indicating the presence of IP receptors on adrenergic neurons, the effects of PGI_2 and selective IP receptor agonist were not examined in the present study.

There have been controversial results concerning the effect of U46619, a stable TXA_2 mimetic, on electrically induced noradrenaline release in various tissues. U46619 caused strong inhibition of noradrenaline release in the rat stomach [39] and moderate inhibition in the rabbit aorta [34] and rat mesenteric bed [38]. On the other hand, U46619 enhanced noradrenaline release in the human isolated iris [35], rabbit vas deference [51] and mesenteric artery [33]. In the porcine uterine longitudinal muscles, U46619 conspicuously decreased the EFS-evoked [^{3}H]-noradrenaline release in a concentration-dependent manner, and the inhibition was comparable to that induced by PGE_2. The inhibition by U46619 was effectively blocked by a TP receptor antagonist

(SQ29548), confirming the involvement of TP receptor activation in the inhibitory responses. Localization of TP receptors on uterine adrenergic nerves was also demonstrated by immunohistochemical study using a TP receptor antibody and TH and/or PGP 9.5 antibodies. However, it was not clear from the present experiments whether TP and EP_3 receptors are expressed on the same adrenergic neurons or not.

As described at a previous section, the S2/S1 ratio of EFS-induced [^{3}H]-noradrenaline release in the presence of indomethacin was significantly higher than that in the absence of indomethacin. Since cyclooxygenase inhibitors decreased uterine PGE_2 and $PGF_{2\alpha}$ contents in the pig [14], the enhancement of EFS-evoked noradrenaline release in the presence of indomethacin might be caused by prevention of presynaptic prostanoid receptor-mediated inhibition of noradrenaline release by endogenous prostanoids, such as PGE_2, $PGF_{2\alpha}$ and TXA_2.

Our previous experiments indicated the presence of contractile prostanoid receptors (EP_1, EP_3, FP, TP) and relaxant prostanoid receptors (EP_2, DP, IP) on smooth muscle cells and indicated that endogenous prostanoids regulate spontaneous contractility of longitudinal muscle of the non-pregnant porcine uterus [11, 12, 14]. In addition to direct regulation of uterine contractility by smooth muscle prostanoid receptors, the present results indicate that prostanoid receptors (EP_3 and TP) indirectly mediate regulation of contractility through affecting noradrenaline release. The porcine uterus has also been shown to be innervated by cholinergic nerves, especially in the circular muscles [20]. Therefore, it would be interesting to investigate whether presynaptic prostanoid receptors are present on uterine cholinergic nerves or not.

In conclusion, prostanoid EP_3 and TP receptors are located on adrenergic neurons of

the longitudinal smooth muscle layer of the porcine uterus, and endogenous prostanoids have inhibitory regulatory roles in release of noradrenaline through these presynaptic receptors. This mechanism could offer important supplemental functional roles of prostanoids-prostanoid receptors in the female reproductive organ, such as modulation of uterine contractility.

Acknowledgement

This work was supported by a grant-in-aid for JSPS fellows (17・05481) from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] Tsuboi, K., Sugimoto, Y., Iwane, A., Yamamoto, K., Yamamoto, S., and Ichikawa, A. Uterine expression of prostaglandin H2 synthase in late pregnancy and during parturition in prostaglandin F receptor-deficient mice. Endocrinology 141(1):315-24, 2000.

[2] Schuster, V.L. Prostaglandin transport. Prostaglandins & Other Lipid Mediators 68-69:633-47, 2002.

[3] Chan, W.Y. Uterine and placental prostaglandins and their modulation of oxytocin sensitivity and contractility in the parturient uterus. Biology of Reproduction 29(3):680-8, 1983.

[4] Romero, R., Munoz, H., Gomez, R.*, et al.* Increase in prostaglandin bioavailability precedes the onset of human parturition. Prostaglandins, Leukotrienes, and Essential fatty acids 54(3):187-91, 1996.

[5] Sugimoto, Y., Yamasaki, A., Segi, E.*, et al.* Failure of parturition in mice lacking the prostaglandin F receptor. Science (New York, NY 277(5326):681-3, 1997.

[6] Davis, B.J., Lennard, D.E., Lee, C.A.*, et al.* Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. Endocrinology 140(6):2685-95, 1999.

[7] Challis, J.R., Sloboda, D.M., Alfaidy, N.*, et al.* Prostaglandins and mechanisms of preterm birth. Reproduction (Cambridge, England) 124(1):1-17, 2002.

[8] Loftin, C.D., Trivedi, D.B., and Langenbach, R. Cyclooxygenase-1-selective inhibition prolongs gestation in mice without adverse effects on the ductus arteriosus. The Journal of Clinical Investigation 110(4):549-57, 2002.

[9] Peri, K.G., Quiniou, C., Hou, X*., et al.* THG113: a novel selective FP antagonist that delays preterm labor. Seminars in Perinatology 26(6):389-97, 2002.

[10] Crankshaw, D.J. Pharmacological techniques for the in vitro study of the uterus. Journal of Pharmacological and Toxicological Methods 45(2):123-40, 2001.

[11] Cao, J., Shayibuzhati, M., Tajima, T., Kitazawa, T., and Taneike, T. In vitro pharmacological characterization of the prostanoid receptor population in the non-pregnant porcine myometrium. European Journal of Pharmacology 442(1-2):115-23, 2002.

[12] Cao, J., Wakatsuki, A., Yoshida, M., Kitazawa, T., and Taneike, T. Thromboxane A2 (TP) receptor in the non-pregnant porcine myometrium and its role in regulation of spontaneous contractile activity. European Journal of Pharmacology 485(1-3):317-27, 2004.

[13] Cao, J., Yosida, M., Kitazawa, T., and Taneike, T. Uterine region-dependent differences in responsiveness to prostaglandins in the non-pregnant porcine myometrium. Prostaglandins & Other Lipid Mediators 75(1-4):105-22, 2005.

[14] Cao, J., Kitazawa, T., Takehana, K., and Taneike, T. Endogenous prostaglandins regulate spontaneous contractile activity of uterine strips isolated from non-pregnant pigs. Prostaglandins & Other Lipid Mediators 81(3-4):93-105, 2006.

[15] Nakanishi, H., and Wood, C. Cholinergic mechanisms in the human uterus. The Journal of Obstetrics and Gynaecology of the British Commonwealth 78(8):716-23, 1971.

[16] Hollingsworth, M. The innervation of the rat cervix and its pharmacology in vitro and in vivo. British Journal of Pharmacology 52(4):539-47, 1974.

[17] Hollingsworth, M. Mechanical responses of rat isolated uterine horns to transmural stimulation. British Journal of Pharmacology 55(1):41-6, 1975.

[18] Elmer, M., Alm, P., and Thorbert, G. Electrical field stimulation of myometrial strips from non-pregnant and pregnant guinea-pigs. Acta Physiologica Scandinavica 108(3):209-13, 1980.

[19] Sato, S., Hayashi, R.H., and Garfield, R.E. Mechanical responses of the rat uterus, cervix, and bladder to stimulation of hypogastric and pelvic nerves in vivo. Biology of Reproduction 40(2):209-19, 1989.

[20] Taneike, T., Miyazaki, H., Nakamura, H., and Ohga, A. Autonomic innervation of the circular and longitudinal layers in swine myometrium. Biology of Reproduction 45(6):831-40, 1991.

[21] Taneike, T., Bando, S., Takasaki, K.*, et al.* Muscle layer and regional differences in autonomic innervation and responsiveness to transmitter agents in swine myometrium. Journal of Autonomic Pharmacology 14(3):213-27, 1994.

[22] Taneike, T., Narita, T., Kitazawa, T., Bando, S., Teraoka, H., and Ohga, A. Binding and functional characterization of alpha-2 adrenoceptors in isolated swine myometrium. Journal of Autonomic Pharmacology 15(2):93-105, 1995.

[23] Thorbert, G., Alm, P., Bjorklund, A.B., Owman, C., and Sjoberg, N.O. Adrenergic innervation of the human uterus. Disappearance of the transmitter and transmitter-forming enzymes during pregnancy. Am J Obstet Gynecol 135(2):223-6, 1979.

[24] Alm, P., and Lundberg, L.M. Co-existence and origin of peptidergic and adrenergic nerves in the guinea pig uterus. Retrograde tracing and immunocytochemistry,

effects of chemical sympathectomy, capsaicin treatment and pregnancy. Cell Tissue Res 254(3):517-30, 1988.

[25] Moustafa, F.A. Changes in cholinergic and noradrenergic nerves in the pregnant and postpartum uterus of the albino rat and guinea pig. Acta Anatomica 132(4):310-6, 1988.

[26] Haase, E.B., Buchman, J., Tietz, A.E., and Schramm, L.P. Pregnancy-induced uterine neuronal degeneration in the rat. Cell Tissue Res 288(2):293-306, 1997.

[27] Zupko, I., Csonka, D., and Falkay, G. A rat model for functional characterization of pregnancy-induced denervation and postpartum reinnervation in the myometrium and cervix: a superfusion study. Reproduction (Cambridge, England) 130(5):743-9, 2005.

[28] Walch, L., de Montpreville, V., Brink, C., and Norel, X. Prostanoid EP(1)- and TP-receptors involved in the contraction of human pulmonary veins. British Journal of Pharmacology 134(8):1671-8, 2001.

[29] De Backer, O., Leclere, P.G., and Lefebvre, R.A. Pharmacological characterization of pre- and postsynaptic prostanoid receptors in pig gastric fundus. Neuropharmacology 45(5):684-90, 2003.

[30] Tilley, S.L., Hartney, J.M., Erikson, C.J.*, et al.* Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. American Journal of Physiology 284(4):L599-606, 2003.

[31] Jadhav, V., Jabre, A., Lin, S.Z., and Lee, T.J. EP1- and EP3-receptors mediate prostaglandin E2-induced constriction of porcine large cerebral arteries. J Cereb Blood Flow Metab 24(12):1305-16, 2004.

[32] Dey, I., Lejeune, M., and Chadee, K. Prostaglandin E2 receptor distribution and function in the gastrointestinal tract. British Journal of Pharmacology 149(6):611-23, 2006.

[33] Trachte, G.J., and Stein, E.A. Thromboxane receptor agonists enhance adrenergic neurotransmission in rabbit isolated mesenteric arteries. The Journal of Pharmacology and Experimental Therapeutics 249(1):216-20, 1989.

[34] Jensen, T.J., and Nedergaard, O.A. Modulation of norepinephrine release from sympathetic neurons of the rabbit aorta by prejunctional prostanoid receptors. The Journal of Pharmacology and Experimental Therapeutics 291(1):7-11, 1999.

[35] Awe, S.O., Opere, C.A., Harris, L.C., Uketui, A.J., and Ohia, S.E. Effect of isoprostanes on sympathetic neurotransmission in the human isolated iris-ciliary body. Neurochemical research 25(4):491-6, 2000.

[36] Gobel, I., Trendelenburg, A.U., Cox, S.L., Meyer, A., and Starke, K. Electrically evoked release of [(3)H]noradrenaline from mouse cultured sympathetic neurons: release-modulating heteroreceptors. Journal of Neurochemistry 75(5):2087-94, 2000.

[37] Opere, C.A., Awe, S.O., Harris, L.C., LeDay, A.M., and Ohia, S.E. Potentiation of sympathetic neurotransmission in bovine isolated irides by isoprostanes. Free Radical Research 35(3):257-64, 2001.

[38] Hoang, D., Macarthur, H., Gardner, A., Yang, C.L., and Westfall, T.C. Prostanoid-induced modulation of neuropeptide Y and noradrenaline release from the rat mesenteric bed. Autonomic & Autacoid Pharmacology 23(2):141-7, 2003.

[39] Yokotani, K., Nakamura, K., and Okada, S. Prostanoid EP3 and TP

receptors-mediated inhibition of noradrenaline release from the isolated rat stomach. European Journal of Pharmacology 459(2-3):187-93, 2003.

[40] McDonald, L.E. Femaile reproductive system. In: *Veterinary endocrinology and reproduction*. (L.E. McDonald, ed.) Lea & Febiger, Philadelphia. 1975. pp. 247-303.

[41] Kitazawa, T., Maezono, Y., and Taneike, T. The mechanisms of α_2-adrenoceptor agonist-induced contraction in longitudinal muscle of the porcine uterus. European Journal of Pharmacology 390(1-2):185-95, 2000.

[42] Queiroz, G., Talaia, C., and Goncalves, J. ATP modulates noradrenaline release by activation of inhibitory P_{2Y} receptors and facilitatory P_{2X} receptors in the rat vas deferens. The Journal of Pharmacology and Experimental Therapeutics 307(2):809-15, 2003.

[43] Kiriyama, M., Ushikubi, F., Kobayashi, T., Hirata, M., Sugimoto, Y., and Narumiya, S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. British Journal of Pharmacology 122(2):217-24, 1997.

[44] Mitchell, B.S., and Ahmed, E. An immunohistochemical study of the catecholamine synthesizing enzymes and neuropeptides in the female guinea-pig uterus and vagina. The Histochemical Journal 24(6):361-7, 1992.

[45] Bae, S.E., Corcoran, B.M., and Watson, E.D. Immunohistochemical study of the distribution of adrenergic and peptidergic innervation in the equine uterus and the cervix. Reproduction (Cambridge, England) 122(2):275-82, 2001.

[46] Serone, A.P., and Angus, J.A. Role of N-type calcium channels in autonomic

neurotransmission in guinea-pig isolated left atria. British Journal of Pharmacology 127(4):927-34, 1999.

[47] Tanaka, Y., Mochizuki, Y., Tanaka, H., and Shigenobu, K. Significant role of neuronal non-N-type calcium channels in the sympathetic neurogenic contraction of rat mesenteric artery. British Journal of Pharmacology 128(7):1602-8, 1999.

[48] Spicuzza, L., Giembycz, M.A., Barnes, P.J., and Belvisi, M.G. Prostaglandin E2 suppression of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by interacting with prostanoid receptors of the EP3-subtype. British Journal of Pharmacology 123(6):1246-52, 1998.

[49] Funk, C.D., Furci, L., FitzGerald, G.A., Grygorczyk, R., Rochette, C., Bayne, M.A., Abramovitz, M., Adam, M., and Metters, K.M. Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. Journal of Biological Chemistry 268(35):26767-26772, 1993.

[50] Narumiya, S., Sugimoto, Y., and Ushikubi, F. Prostanoid receptors: structures, properties, and functions. Physiological Reviews 79(4):1193-226, 1999.

[51] Trachte, G.J. Thromboxane agonist (U46619) potentiates norepinephrine efflux from adrenergic nerves. The Journal of Pharmacology and Experimental Therapeutics 237(2):473-7, 1986.

FIGURE LEGENDS

Fig. 1. Frequency-dependent [^{3}H]-noradrenaline release induced by EFS in longitudinal smooth muscle of the non-pregnant porcine uterus in the absence (○) and presence (●) of indomethacin (3 μM). Ordinate: relative outflow of [^{3}H]-noradrenaline expressed as a percent of total radioactivity of the tissue in the fraction period. Abscissa: fraction period (5 min) and EFS frequency (Hz). Each point represents the mean ± S.E.M. of more than six experiments. *, significantly different ($p<0.05$) compared with the corresponding values in the absence of indomethacin.

Fig. 2. Pharmacological characteristics of EFS-evoked noradrenaline release. Each figure shows a comparison of noradrenaline release at the first stimulation (S1; 10 Hz) and that at the second stimulation (S2; 10 Hz). EFS-induced noradrenaline release was reproducible in the control (A), but tetrodotoxin (1 μM; B) and ω-conotoxin GVIA (1 μM; C) treatment (arrows) inhibited the stimulation-evoked [^{3}H]-outflow. EFS-induced noradrenaline release was also abolished in the Ca^{2+}-free incubation medium containing EGTA (1 mM; D). Ordinate: relative outflow of [^{3}H]-noradrenaline expressed as a percent of total radioactivity of the tissue in the fraction period. Abscissa: fraction period (5 min). Each point represents the mean ± S.E.M. of more than four experiments.

Fig. 3. Effects of PGE_2, $PGF_{2\alpha}$ and PGD_2 on EFS-induced [^{3}H]-noradrenaline release in longitudinal muscle of the non-pregnant porcine uterus. The uterine muscle strips were stimulated (10 Hz for 30 s) two times (S1 and S2) at a 30-min interval. Before

application of S2 stimulation, muscle strips were treated with 1 μM of PGE_2 (A), $PGF_{2\alpha}$ (B) and PGD_2 (C) for 2.5 min (black bar) and S2 was applied in the presence of prostanoids (filled symbols). Open symbols are results of the time-matched control without prostaglandins treatment. Ordinate: relative outflow of [^{3}H]-noradrenaline expressed as a percent of total radioactivity of the tissue in the fraction period. Abscissa: fraction period (5 min). The effects of prostanoids on EFS-induced [^{3}H]-noradrenaline release are also indicated using S2/S1 ratio (D). Each value is the mean ± S.E.M. of more than six experiments. * P<0.05, ** P<0.01 compared with control.

Fig. 4. Effects of EP receptor agonists and antagonists on EFS-induced [^{3}H]-noradrenaline release in longitudinal muscle of the non-pregnant porcine uterus. (A) Sulprostone decreased the S2/S1 ratio in a concentration-dependent manner (1 nM-1 μM). (B) Butaprost (1 μM) did not inhibit noradrenaline release. Effects of SC19220 (10 μM) itself on the [^{3}H]-noradrenaline release and on the inhibition by PGE_2 and sulprostone were examined. Each value is the mean ± S.E.M. of more than four experiments. **, P<0.01 compared with control. NS indicates not significant difference.

Fig. 5. Effects of $PGF_{2\alpha}$ (1 μM) and fluprostenol (FP receptor agonist; 1 μM) on EFS-induced release of [^{3}H]-noradrenaline from longitudinal muscle strips of the non-pregnant porcine uterus. Each value is the mean ± S.E.M. of more than six experiments. **, P<0.01 compared with control.

Fig. 6. Effects of U46619 on stimulation-induced [^{3}H]-noradrenaline release and

inhibition of U46619-induced action by SQ29548 in longitudinal muscle strips of the non-pregnant porcine uterus. (A) Concentration-dependent inhibitory effects of U46619 (1 nM – 1 μM). (B) SQ29548 significantly recovered the inhibitory responses of two different concentrations of U46619 (100 nM and 1 μM), but SQ29548 itself did not affect the EFS-induced release. Each value is the mean ± S.E.M. of more than four experiments. * $P<0.05$, ** $P<0.01$ compared with control. # $P<0.05$ compared with U46619 alone.

Fig. 7. EP_3 receptor expression on adrenergic neurons in the longitudinal muscle layer of the non-pregnant porcine uterus (double immunofluorescence staining). EP_3 receptors were observed both in smooth muscle layers and fiber-like structures (A and D, arrow). PGP 9.5 (B) or TH immunoreactive (E) helical fibers (green) were scattered among longitudinal muscle layers. Yellow color on merged photographs (C, F) indicates the expression of EP_3 receptors on neural elements (C) and adrenergic fibers (F). Scale bar = 20 μm.

Fig. 8. TP receptor expression on adrenergic neurons in the longitudinal muscle layer of the non-pregnant porcine uterus (double immunofluorescence staining). TP receptors were observed both in smooth muscle layers and fiber-like structures (A and D, arrow). PGP 9.5 (B) or TH immunoreactive (E) helical fibers (green) were oriented in the same direction in the muscle layers. Ψελλοω χολορ ον μεργεδ πηοτογραπησ (Χ, Φ) ινδιχατεσ τηε εξπρεσσιον οφ ΤΠ ρεχεπτορσ on neural elements (C) and adrenergic fibers (F). Scale bar = 20 μm.

Fig. 1.

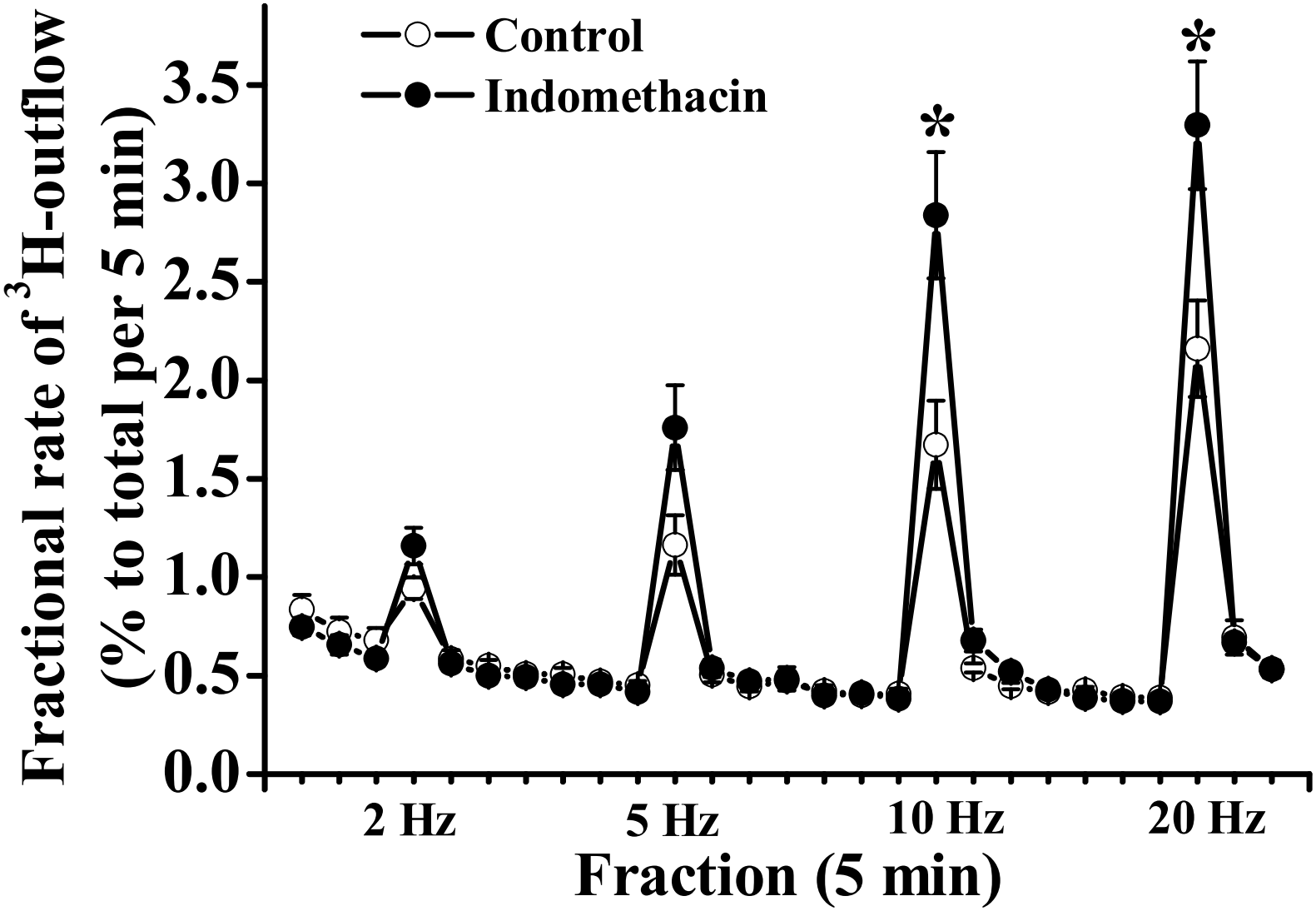

Control
Indomethacin
Fractional rate of ^{3}H-outflow
(% to total per 5 min)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
*
*
2 Hz
5 Hz
10 Hz
20 Hz
Fraction (5 min)

Fig. 2.

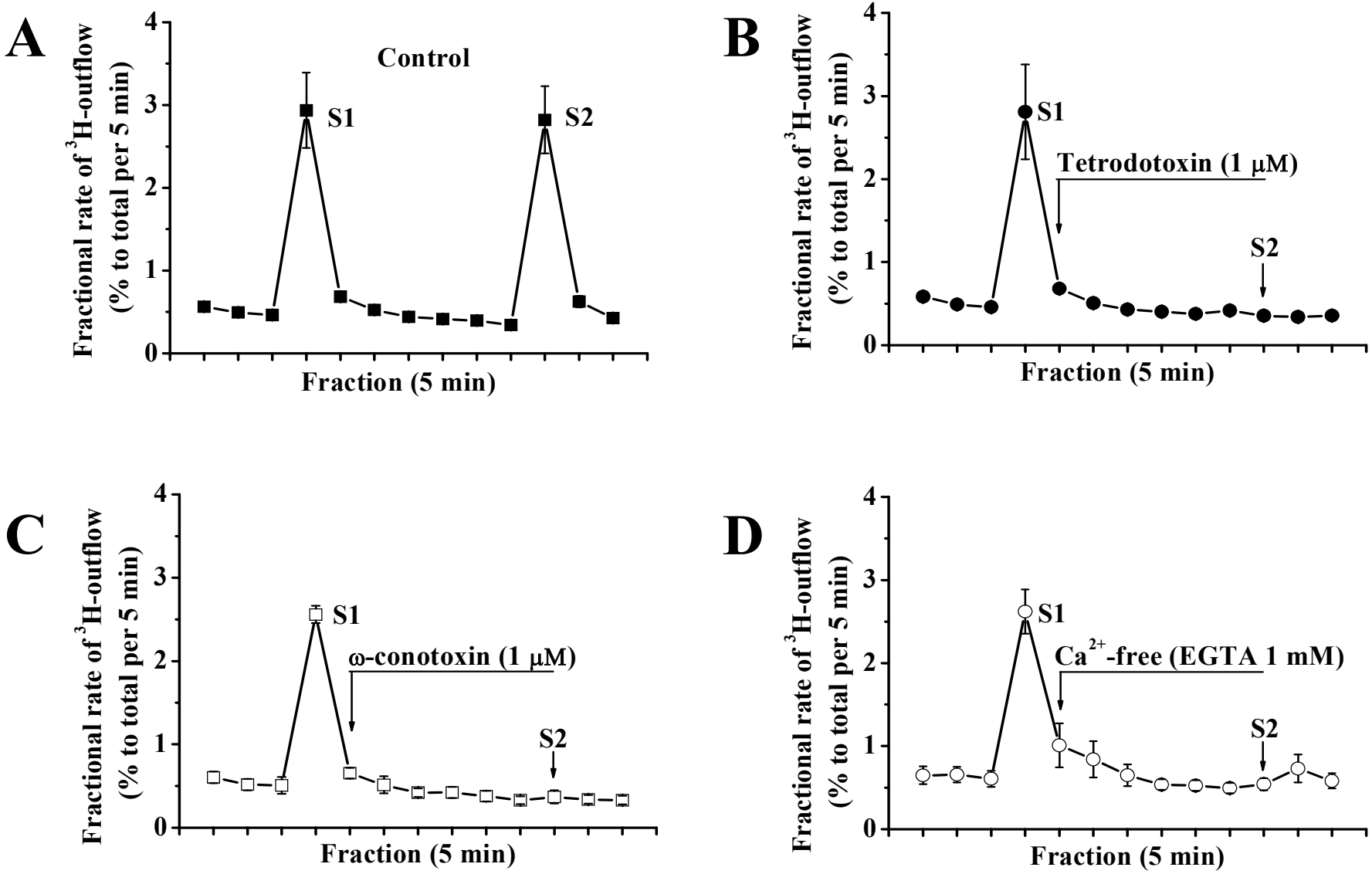
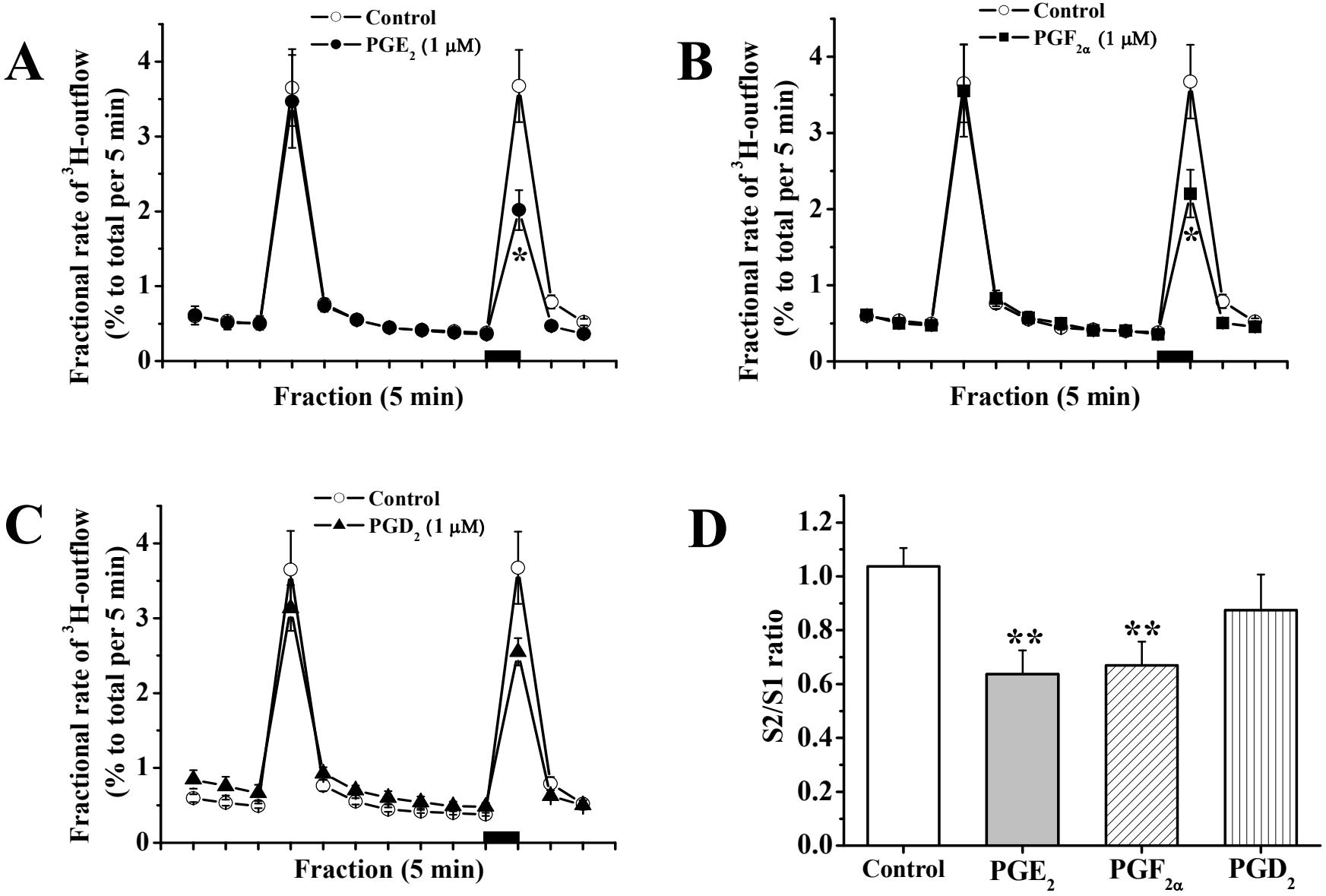

A
Control
S1
S2
Fractional rate of ^{3}H-outflow (% to total per 5 min)
Fraction (5 min)
B
S1
Tetrodotoxin (1 μM)
S2
Fractional rate of ^{3}H-outflow (% to total per 5 min)
Fraction (5 min)
C
S1
ω-conotoxin (1 μM)
S2
Fractional rate of ^{3}H-outflow (% to total per 5 min)
Fraction (5 min)
D
S1
Ca^{2+}-free (EGTA 1 mM)
S2
Fractional rate of ^{3}H-outflow (% to total per 5 min)
Fraction (5 min)

Fig. 3.


A
Control
PGE2 (1 μM)
Fractional rate of 3H-outflow (% to total per 5 min)
Fraction (5 min)
B
Control
PGF2α (1 μM)
Fractional rate of 3H-outflow (% to total per 5 min)
Fraction (5 min)
C
Control
PGD2 (1 μM)
Fractional rate of 3H-outflow (% to total per 5 min)
Fraction (5 min)
D
S2/S1 ratio
Control
PGE2
PGF2α
PGD2

Figure(s)

Fig. 4.

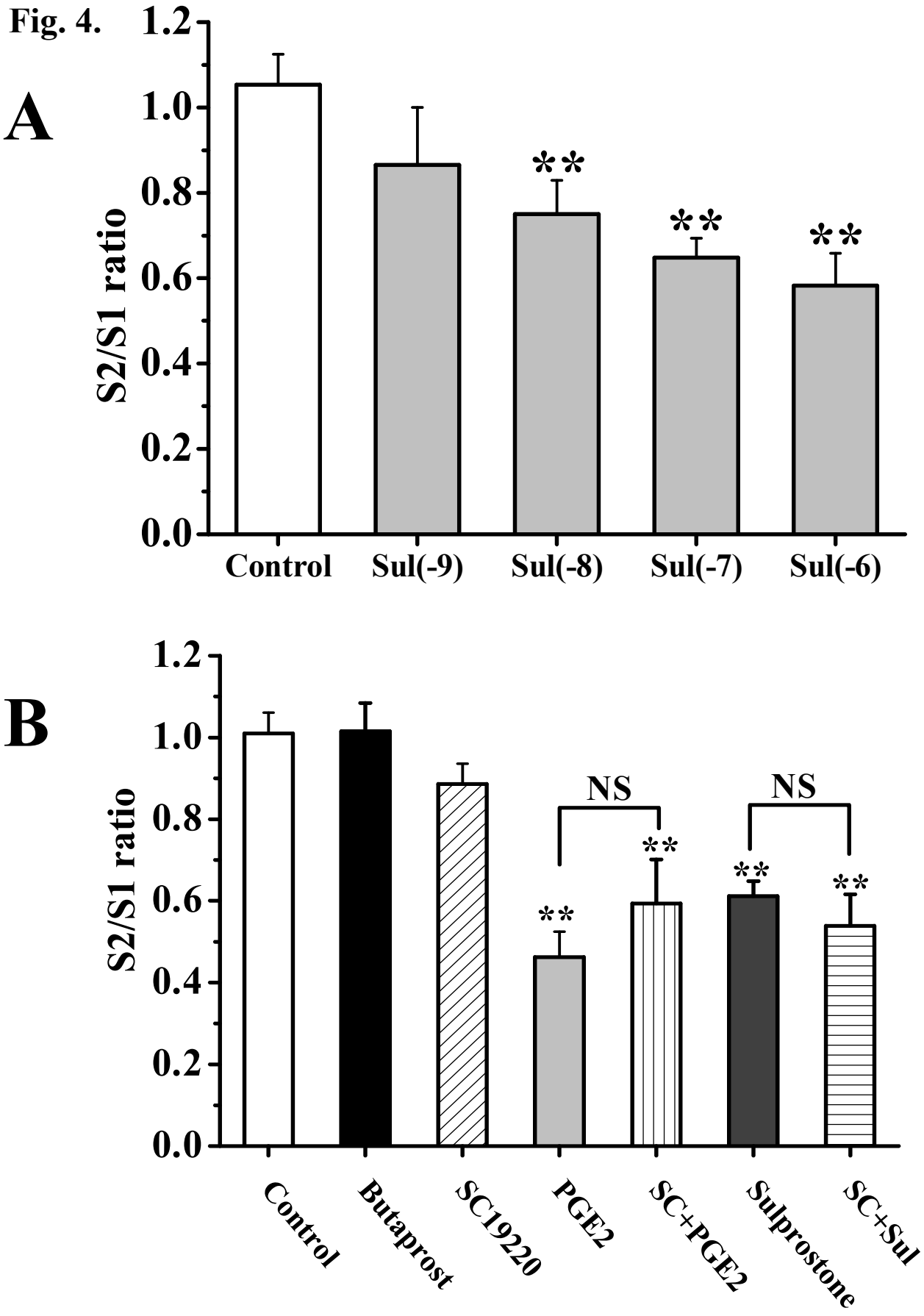


Fig. 5.

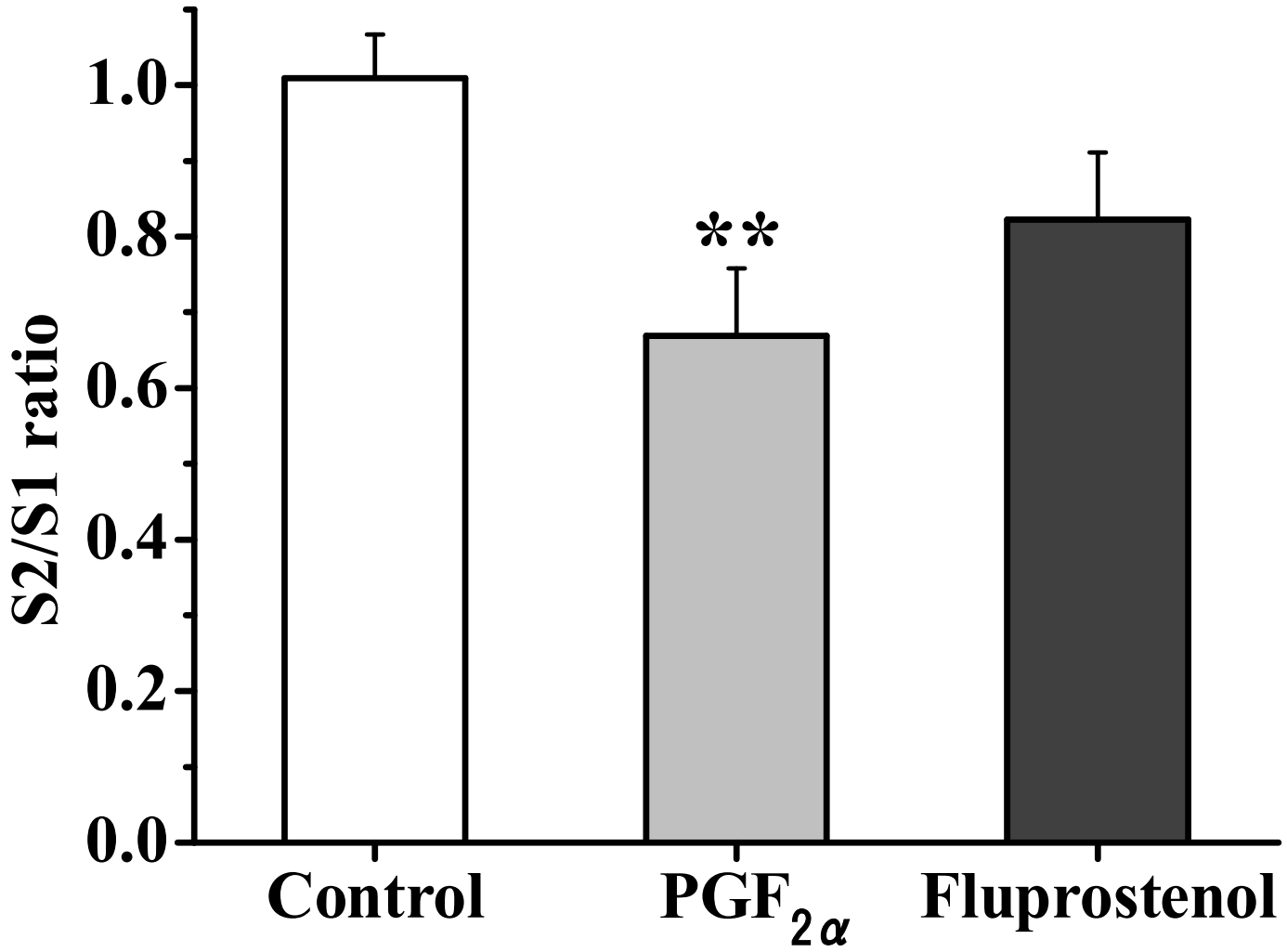
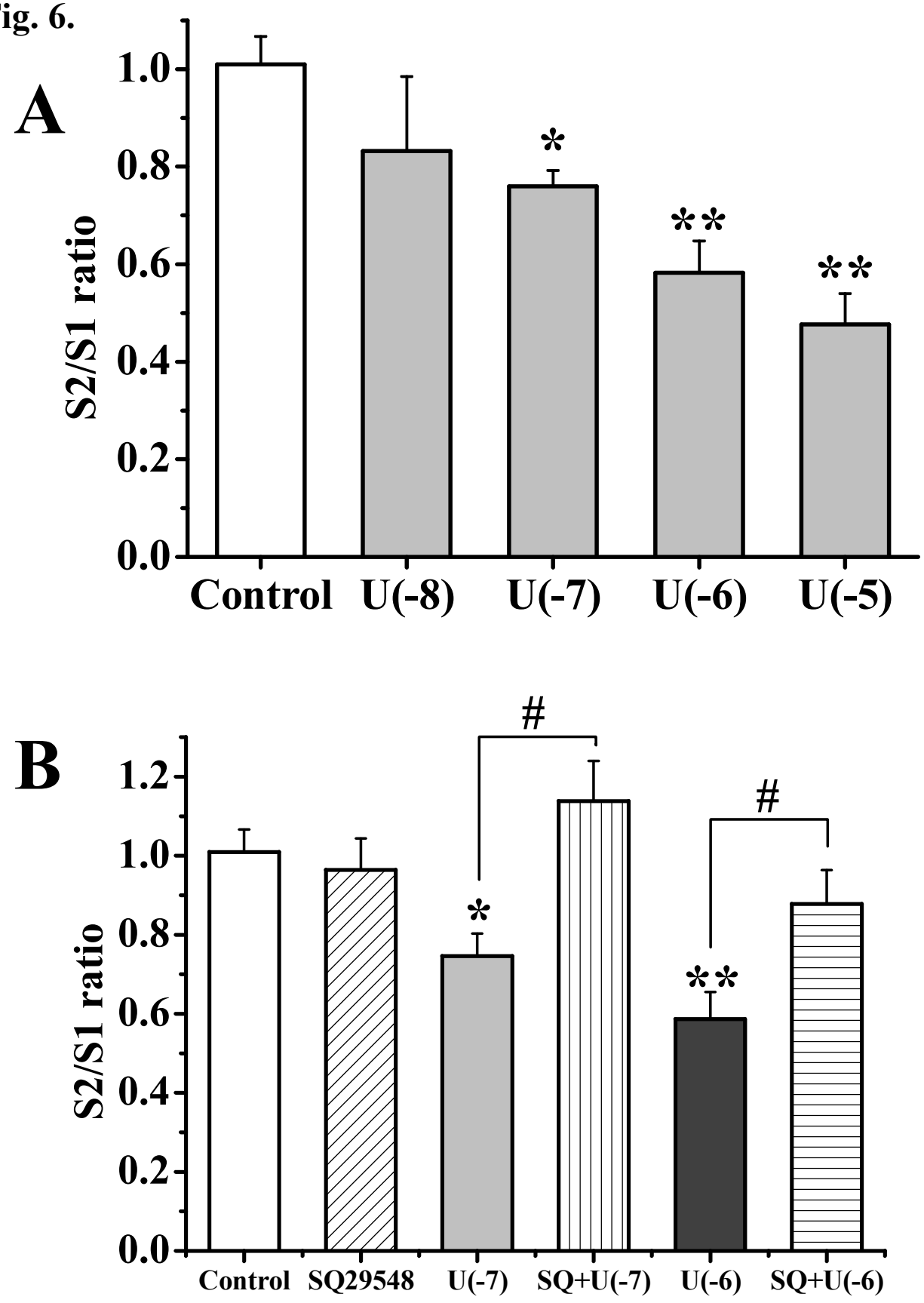
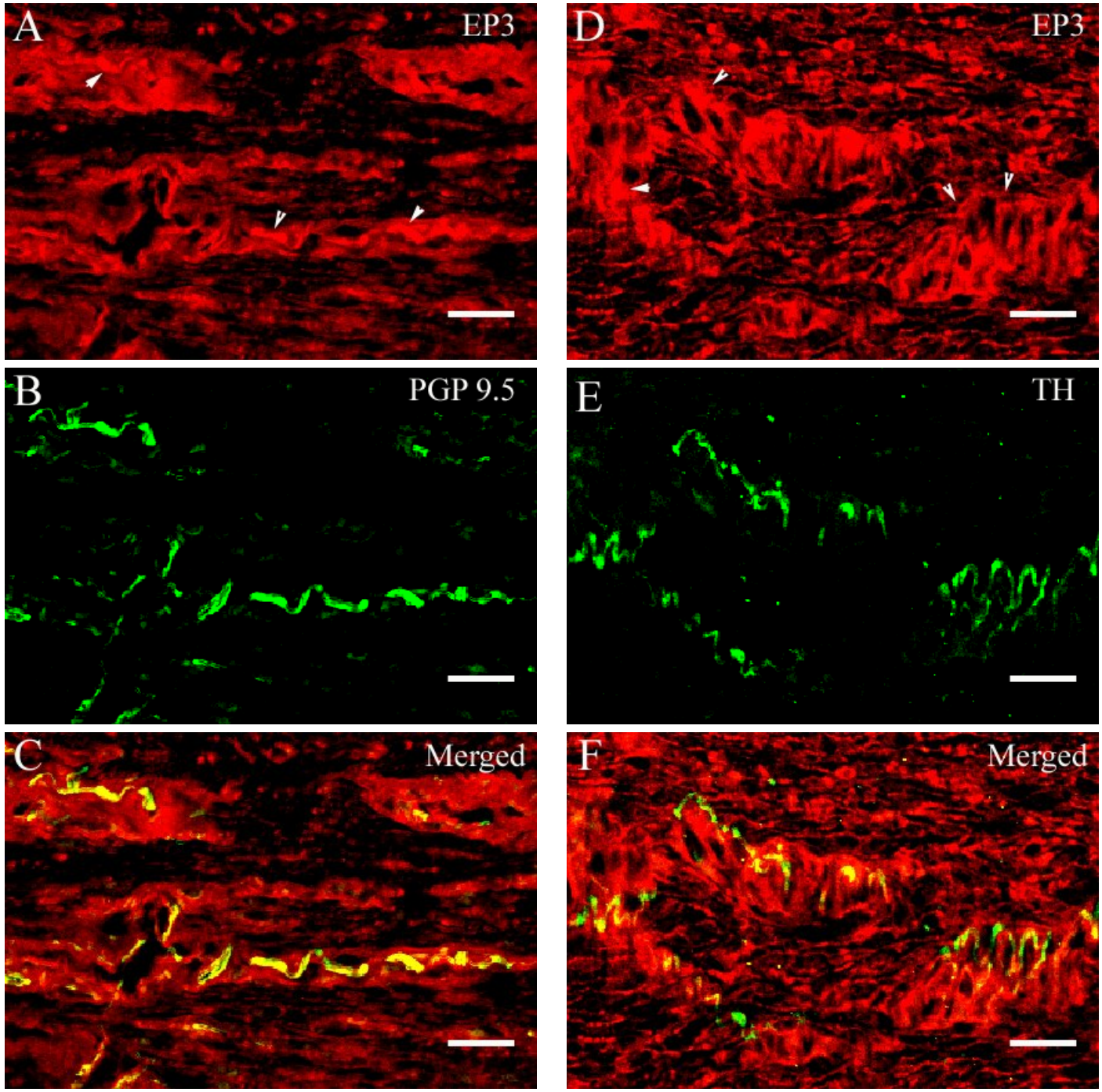


Fig. 6.


A
S2/S1 ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
Control
U(-8)
U(-7)
U(-6)
U(-5)
B
S2/S1 ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
#
*
**
Control
SQ29548
U(-7)
SQ+U(-7)
U(-6)
SQ+U(-6)

Figure(s)

Fig. 7.



Figure(s)

Fig. 8.