Full Paper

Endothelin ET_A- and ET_B-Receptor-Mediated Inhibition of Noradrenaline Release From Isolated Rat Stomach

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Abstract. We examined the effect of endothelin-1, endothelin-3 and sarafotoxin S6c on the release of noradrenaline from gastric sympathetic nerve terminals using an isolated, vascularly perfused rat stomach. The release of noradrenaline evoked by electrical stimulation of the gastric postganglionic sympathetic nerves (at 2.5 Hz for 1 min) was inhibited by endothelin-1 (10^{-10} – 10^{-8} M), endothelin-3 (10^{-9} – 10^{-8} M) and sarafotoxin S6c (a highly selective agonist of endothelin ET_B receptors) (10^{-9} – 10^{-8} M) in a concentration-dependent manner; the inhibitory potencies were as follows: endothelin-1 > endothelin-3 > sarafotoxin S6c. The inhibitory effect of endothelin-1 (3×10^{-9} M) on noradrenaline release was abolished by BQ-123 (a selective antagonist of endothelin ET_A receptors) in a dose-dependent manner (10^{-7} and 10^{-6} M), but not influenced by BQ-788 (a selective antagonist of endothelin ET_B receptors) (10^{-7} and 10^{-6} M). The endothelin-1-induced inhibition of noradrenaline release was attenuated by pretreatment with pertussis toxin ($10 \mu\text{g}/\text{animal}$, i.v., 4 days before experiments), but not influenced by indomethacin (3×10^{-6} M). These results indicate that endothelin ET_A and ET_B receptors located on the sympathetic nerve terminals play a role in the inhibition of noradrenaline release from the rat stomach: endothelin ET_A receptor-mediated inhibition is carried out by pertussis toxin-sensitive and indomethacin-insensitive mechanisms.

Keywords: endothelin, release of noradrenaline, rat stomach, endothelin ET_A receptor, endothelin ET_B receptor

Introduction

Endothelin, discovered as a potent vasoactive peptide produced by cultured endothelial cells (1), is a family of peptides, endothelin-1, endothelin-2, and endothelin-3 (2, 3). Sarafotoxin S6c, isolated from the venom of the snake *Atractaspis engaddensis*, possesses structural features and pharmacological activity similar to those of endothelins (4, 5). Endothelins exert pharmacological actions such as prolonged contraction of vascular, intestinal, uterine, bronchial, and other smooth muscle (6–8), and they may play a certain physiological and/or pathophysiological role in these organs (9–11). Besides their potent direct constrictor actions, these peptides may exert indirect control of constrictive tone via modulation of sympathetic neurotransmission. Indeed, endothelin-1

has been shown to reduce noradrenaline release in some sympathetically innervated tissues such as the rat mesenteric artery (12), guinea pig femoral artery, ileum and vas deferens (13–15), and dog kidney (16).

The presence of endothelin has been demonstrated in the rat gastrointestinal tract (17) and in the enteric neurons of the human gut (18, 19). Endothelin binding sites have also been identified in the rat intestine (17) and guinea pig ileum (20). However, little is known about the role of endothelin on the gastric sympathetic neurotransmission. In the present experiment, therefore, we examined the effects of endothelin-1, endothelin-3, and sarafotoxin S6c on the release of noradrenaline from rat gastric sympathetic nerve terminals.

Materials and Methods

Perfusion experiments

Male Wistar rats (Shizuoka Laboratory Animal Center,

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Hamamatsu, Japan) weighing about 350 g were fasted overnight before experiments. The isolated, vascularly perfused stomach preparations were made as described previously (21–23). Briefly, under urethane anesthesia, the abdomen was opened with a midline incision. After ligation of the abdominal aorta just above the branching of the celiac artery, the cannula was inserted into the celiac artery via an incision placed on the aorta, and modified Krebs-Ringer solution (pH 7.4) bubbled with a mixture of 95% O₂ and 5% CO₂ maintained at pH 7.4 was perfused with a constant flow rate of 2.5 ml per min. Modified Krebs-Ringer solution was composed of 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl₂, 1.1 mM MgCl₂, 1.1 mM NaH₂PO₄, 25 mM NaHCO₃, 11.1 mM glucose, 0.05% of bovine serum albumin, 10 mM pargyline, and 1 mM phentolamine. The tube was inserted into the lumen of the stomach via a pylorus ring to drain the contents of the stomach throughout the experiment. The esophagus, duodenum, spleen, and pancreas were dissected after ligation of the vessels, and the vascularly perfused stomach was kept in a chamber prewarmed at 37°C. Each 2-min effluent from the portal vein was collected in chilled tubes containing 0.5 ml of 4 N perchloric acid, 1 ng of 3,4-dihydroxybenzylamine as an internal standard, and one drop of 2% sodium pyrosulfite solution.

After an equilibration period of 60 min, the first electrical stimulation consisting of square-wave pulses (2.5 Hz, 2-ms duration, 10 mA for 1 min) was applied to the periarterial nerves around the left gastric artery, which contain the postganglionic sympathetic nerves, using bipolar electrodes. The second electrical stimulation was carried out 26 min after the first stimulation. Perfusion medium containing test substances was changed 14 min before the second electrical stimulation.

In some experiments, rats were pretreated with pertussis toxin (10 µg per rat dissolved in 0.1 ml of sodium phosphate-buffered saline, pH 7.0, 4 days before experiments) or vehicle injected into the dorsal penic vein under a light ether anesthesia, as described in our previous paper (24).

Noradrenaline assay in the medium and the stomach

At the end of each experiment, the stomach was homogenized in 20 ml of 0.4 N perchloric acid containing 18.6 mg of disodium EDTA, 200 µl of 2% sodium pyrosulfite solution, and 500 ng of 3,4-dihydroxybenzylamine as an internal standard. The homogenate was centrifuged for 10 min at 14,000 ×g at 4°C. The supernatant was analyzed to determine the tissue level of noradrenaline.

Catecholamines in the effluent and the supernatant of tissue homogenate were extracted by the method of

Anton and Sayre (25), with a slight modification, and were assayed electrochemically using high-performance liquid chromatography (26). Specifically, 2 ml of effluent or an aliquot (0.1 ml) of supernatant was transferred to a centrifuge tube containing 30 mg of activated alumina and 3 ml of 1.5 M Tris Buffer (pH 8.6) containing 0.1 M disodium EDTA dihydrate, after which the preparations were shaken for 10 min. The supernatant was discarded and the alumina was washed three times with double-deionized water, and catecholamines were eluted with 500 µl of 4% of acetic acid containing 0.1 mM disodium EDTA.

The high-performance liquid chromatography-electrochemical detection system consisted of a solvent delivery system (Model PU-980; Japan Spectroscopic Co., Ltd., Tokyo), a sample processor (Model 851-AS, Japan Spectroscopic Co., Ltd.), an ODS column (Cosmosil_{SC18}; Nacalai Tesque, Inc., Kyoto) and an electrochemical detector (Model ECD-300; Eicom, Kyoto) equipped with a graphite electrode. The solvent system consisted of 100 mM NaH₂PO₄-Na₂HPO₄, 0.13 mM disodium EDTA, 8.8 mM sodium octanesulfonate, and 16% methanol. By this assay, 2 pg of noradrenaline could be determined accurately.

Evaluation and statistical analysis

Spontaneous and evoked release of noradrenaline is expressed as a percentage of its tissue content per 2 min. Basal release of noradrenaline was calculated by averaging the amount of noradrenaline released in two subsequent samples before electrical stimulation. The amounts of the evoked release of noradrenaline above the basal level during 12 min after the first or second electrical stimulation is expressed as S₁ or S₂. The effects of test substances are expressed as the ratio of S₂ to S₁. All values are expressed as the means ± S.E.M.

All data were analyzed by repeated-measure analysis of variance, followed by post-hoc analysis with the Bonferroni method for comparing a control with all other means in Figs. 2 and 3. Student *t*-test was used for evaluating the significant difference between two values in Fig. 1 and Table 1. *P* values of less than 0.05 were taken to indicate statistical significance.

Drugs

The following drugs were used: 3,4-dihydroxybenzylamine hydrobromide, indomethacin, pargyline hydrochloride, pertussis toxin, and phentolamine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); cyclo (D- α -aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl) (BQ-123), N-[N-[N-[(2,6-dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl]-1-methoxycarbonyl]-D-tryptophyl]-D-norleucine monosodium (BQ-788) (Research

Biochemicals International, Natick, MA, USA); endothelin-1, endothelin-3, and sarafotoxin S6c (Peptide Institute, Inc., Osaka). All other reagents were the highest grade available (Nacalai Tesque).

Results

Effects of endothelin-1, endothelin-3, and sarafotoxin S6c on the electrically evoked release of noradrenaline from the isolated rat stomach

The amount of noradrenaline remaining in the stomach was 754 ± 10 ng ($n = 147$). Spontaneous release of noradrenaline was about 0.03% of its tissue content per 2 min. Electrical stimulation of the gastric sympathetic nerves at 2.5 Hz for 1 min evoked an increase of noradrenaline release, and this increase rapidly declined toward the basal level (Fig. 1). Repetitive stimulations evoked consistent and reproducible increases in noradrenaline release (S_2/S_1 ratio: 1.04 ± 0.05 , $n = 6$) (Figs. 1 and 2).

After the first stimulation of the gastric sympathetic nerves, the medium was changed to the next one containing endothelin-1 (10^{-10} – 10^{-8} M), endothelin-3 (10^{-9} – 10^{-8} M), or sarafotoxin S6c (10^{-9} – 10^{-8} M). Basal release

Fig. 1. Effect of endothelin-1 on the evoked release of noradrenaline from the isolated rat stomach. Periaortic nerves of the left gastric artery were electrically stimulated twice. The first stimulation was carried out in the normal medium and the second stimulation was carried out in the medium containing endothelin-1 (ET-1) (3×10^{-9} M). The release of noradrenaline (NA) is expressed as % of its tissue content per 2 min. E.S., electrical stimulation of the gastric sympathetic nerves at 2.5 Hz for 1 min. Values are means $\pm$ S.E.M. *Significant difference ($P < 0.05$) from the values of the vehicle-treated control. Open circle, control group ($n = 6$); closed circle, endothelin-1 (3×10^{-9} M)-treated group ($n = 6$).

of noradrenaline was not influenced by these peptides. Endothelin-1, endothelin-3, and sarafotoxin S6c effectively and concentration-dependently reduced the evoked

Fig. 2. Effects of endothelin-1, endothelin-3, and sarafotoxin S6c on the electrically evoked release of noradrenaline from the isolated rat stomach. Endothelin-1 (ET-1) (10^{-10} – 10^{-8} M), endothelin-3 (ET-3) (10^{-9} – 10^{-8} M), or sarafotoxin S6c (10^{-9} – 10^{-8} M) was added during the second electrical stimulation. The effects of these peptides are expressed as the S_2/S_1 ratio. Graph A: Control (C) ($n = 6$), 10^{-10} ET-1 ($n = 4$), 10^{-9} ET-1 ($n = 5$), 3×10^{-9} ET-1 ($n = 6$), 10^{-8} ET-1 ($n = 5$); Graph B: Control (C) (cited from graph A), 10^{-9} ET-3 ($n = 4$), 3×10^{-9} ET-3 ($n = 5$), 10^{-8} ET-3 ($n = 5$); Graph C: Control (C) (cited from graph A), 10^{-9} sarafotoxin S6c ($n = 4$), 3×10^{-9} sarafotoxin S6c ($n = 4$), 10^{-8} sarafotoxin S6c ($n = 7$). *Significant difference ($P < 0.05$) from the control. Other conditions were the same as those in Fig. 1.

release of noradrenaline; the inhibitory potencies of these peptides were as follows: endothelin-1 > endothelin-3 > sarafotoxin S6c (Fig. 2).

Effects of BQ-123 and BQ-788 on the endothelin-1-induced inhibition of noradrenaline release from the isolated rat stomach

We examined the effects of BQ-123 (a selective blocker of endothelin ET_A receptors) and BQ-788 (a selective blocker of endothelin ET_B receptors) on the endothelin-1-induced inhibition of noradrenaline release from the gastric sympathetic nerves (Table 1). The basal and the electrically evoked release of noradrenaline was not affected by BQ-123 (10^{-7} and 10^{-6} M) or BQ-788 (10^{-7} and 10^{-6} M). The inhibitory effect of 3×10^{-9} M endothelin-1 on the evoked release of noradrenaline was attenuated by BQ-123 in a concentration-dependent manner (10^{-7} and 10^{-6} M), while BQ-788 (10^{-7} and 10^{-6} M) had no effect on the inhibitory effect of endothelin-1 on the evoked release of noradrenaline (Table 1).

Effects of indomethacin and pertussis toxin on the endothelin-1-induced inhibition of noradrenaline release from the isolated rat stomach

In the first series of experiments, the stomach was treated with indomethacin (3×10^{-6} M) for 2 h (this treatment was started 1 h before experiments and continued throughout the experiments). The basal release and evoked release of noradrenaline were not influenced by this treatment. The inhibitory effects of endothelin-1 (3×10^{-9} and 10^{-8} M) were not influenced by treatment

with indomethacin (Fig. 3A).

In the second series of experiments, rats were pretreated with pertussis toxin ($10 \mu\text{g}$ per rat, i.v., 4 days before experiments) or vehicle. The basal release of noradrenaline was not influenced; however, the electrically evoked release of noradrenaline was slightly, but not significantly, reduced by pretreatment with this toxin. The inhibitory effects of endothelin-1 (10^{-9} and 3×10^{-9} M) on the evoked release of noradrenaline was

Fig. 3. Effects of indomethacin and pertussis toxin on the endothelin-1-induced inhibition of noradrenaline release from the isolated rat stomach. Graph A: indomethacin (IND) (3×10^{-6} M) was administered throughout the experiment. ET-1 (3×10^{-9} and 10^{-8} M) was administered during the second electrical stimulation. Vehicle-treated control group: control ($n = 6$), 3×10^{-9} M ET-1 ($n = 6$), 10^{-8} M ET-1 ($n = 5$) (cited from Fig. 2); IND-treated group: control ($n = 6$), 3×10^{-9} M ET-1 ($n = 5$), 10^{-8} M ET-1 ($n = 5$). Graph B: pertussis toxin (PTX) ($10 \mu\text{g}/\text{animal}$, i.v.) or vehicle (phosphate-buffered saline) ($10 \mu\text{l}/\text{animal}$, i.v.) was administered 4 days before the experiments. ET-1 (10^{-9} and 3×10^{-9} M) was administered during the second electrical stimulation. Vehicle-treated control group: control ($n = 4$), 10^{-9} M ET-1 ($n = 5$), 3×10^{-9} M ET-1 ($n = 5$); PTX-treated group: control ($n = 4$), 10^{-9} M ET-1 ($n = 4$), 3×10^{-9} M ET-1 ($n = 5$). *Significant difference ($P < 0.05$) from the control. Other conditions were the same as those in Figs. 1 and 2.

Table 1. Effects of BQ-123 and BQ-788 on the endothelin-1-induced inhibition of the evoked release of noradrenaline from the isolated rat stomach

Blocker	ET-1 (3×10^{-9} M)	Number	S ₂ /S ₁ ratio
(-)	(-)	6	1.04 ± 0.05^a
(-)	ET-1	6	0.38 ± 0.07^a
BQ-123 (10^{-7} M)	(-)	4	1.11 ± 0.04
BQ-123 (10^{-6} M)	(-)	6	0.96 ± 0.06
BQ-123 (10^{-7} M)	ET-1	5	0.57 ± 0.09^b
BQ-123 (10^{-6} M)	ET-1	6	0.89 ± 0.02
BQ-788 (10^{-7} M)	(-)	4	0.95 ± 0.12
BQ-788 (10^{-6} M)	(-)	4	1.01 ± 0.02
BQ-788 (10^{-7} M)	ET-1	5	0.31 ± 0.04^b
BQ-788 (10^{-6} M)	ET-1	6	0.32 ± 0.04^b

The periaarterial nerves around the left gastric artery were electrically stimulated twice at 2.5 Hz for 1 min. Endothelin-1 (ET-1) (3×10^{-9} M) and/or BQ-123, ET-1 and/or BQ-788 were administered during the second electrical stimulation. ^acited from Fig. 1. ^bSignificant difference ($P < 0.05$) from the respective control with BQ-123 or BQ-788 alone. Other conditions were the same as those in Figs. 1 and 2.

significantly attenuated by pretreatment with this toxin (Fig. 3B).

Discussion

The effects of endothelins are thought to be mediated by at least two kinds of endothelin receptors, ET_A and ET_B receptors (9, 27). Endothelin ET_A receptors have been shown to bind endothelin-1 with higher affinity than endothelin-3 (28), while endothelin ET_B receptors bind the endothelin-1 and endothelin-3 with equal affinity (29, 30). Endothelins-induced modulation of noradrenaline release from the rat tail artery has already been examined in relation to the endothelin receptor subtypes by using endothelin-1, endothelin-3, and sarafotoxin S6c (31). In this experiment, dual (inhibitory and facilitatory) effects of endothelins and sarafotoxin S6c have been shown on the evoked release of noradrenaline; endothelin-1 (10^{-9} – 3×10^{-8} M) and sarafotoxin S6c [a highly selective agonist of endothelin ET_B receptors (32, 33)] (10^{-12} – 3×10^{-10} M) reduced the evoked release of noradrenaline, while endothelin-3 (3×10^{-7} M) and sarafotoxin S6c (10^{-9} – 10^{-8} M) caused a significant augmentation of the evoked release of noradrenaline. In the present experiment, endothelin-1, endothelin-3, and sarafotoxin S6c effectively inhibited the evoked release of noradrenaline from the gastric sympathetic nerves with the following potencies: endothelin-1 > endothelin-3 > sarafotoxin S6c. From these results, a question has arisen about whether endothelin ET_A and/or ET_B receptors are involved in the inhibition of noradrenaline release from the gastric sympathetic nerves.

BQ-123 has been shown to be a selective antagonist of endothelin ET_A receptors (34), while BQ-788 to be a selective antagonist of the endothelin ET_B receptors (35). In the experiment with rat tail artery described above, endothelin-1-induced inhibition of the electrically evoked release of noradrenaline was resistant to BQ-123 but was prevented by BQ-788, indicating the endothelin ET_B receptor-mediated inhibition of noradrenaline release (31). In the present experiments with the rat stomach, however, BQ-123 concentration-dependently attenuated the endothelin-1-induced inhibition of the evoked release of noradrenaline but BQ-788 had no effect, suggesting the involvement of endothelin ET_A receptors in the endothelin-1-induced inhibition of noradrenaline release from the rat stomach. In addition, endothelin ET_B receptors also play a role in inhibition of noradrenaline release from the gastric sympathetic nerves, since a highly selective endothelin ET_B-receptor agonist sarafotoxin S6c (32, 33) slightly, but significantly, inhibited the release of noradrenaline from the stomach. The co-existence of endothelin ET_A and ET_B

receptors has also been shown in the rat tracheal smooth muscle to inhibit acetylcholine release (36).

The transduction of extracellular signals to specific intracellular events often involves the participation of regulatory GTP-binding proteins. Pertussis toxin has been shown to ADP-ribosylate the α subunit of some GTP-binding proteins (G_i or G_o), resulting in their inactivation (37, 38). Since the pretreatment with pertussis toxin ($10 \mu\text{g}/\text{rat}$, for 4 days) abolished the negative chronotropic and inotropic effects of oxotremorine (a muscarinic receptor agonist) on the isolated, spontaneously beating rat atria preparation (24), pretreatment with the same dose of this toxin seems to be enough to block pertussis toxin-sensitive GTP-binding proteins in the rat stomach. In the present experiment, endothelin-1-induced inhibition of the evoked release of noradrenaline was attenuated by pertussis toxin, suggesting the involvement of these toxin-sensitive GTP-binding proteins in the endothelin ET_A receptor-mediated inhibition of noradrenaline release from the gastric sympathetic nerve terminals. Pertussis toxin has been shown to block endothelin-1-induced contraction of porcine coronary artery (39) and endothelin-1-induced inhibition of the L-type Ca^{2+} current in human atrial myocytes (40). From these results, it is suggested that endothelin-1-induced inhibition of noradrenaline release is due to the attenuation of N-type calcium channels located on the gastric sympathetic nerve terminals (41) through pertussis toxin-sensitive GTP-binding proteins.

Endothelins have been shown to activate phospholipase A₂ which results in the production of prostaglandin E₂, prostacyclin, and thromboxane A₂ in bovine endothelial cells, cultured vascular smooth muscle cells, and rat aorta (42–44). Therefore, in the present experiment, we examined the effect of indomethacin, an inhibitor of cyclooxygenase, on the endothelin-1-induced inhibition of noradrenaline release from the gastric sympathetic nerves. However, the endothelin-1-induced inhibitory effect of noradrenaline release was not attenuated by indomethacin, indicating that a cyclooxygenase-dependent pathway is not involved in the endothelin ET_A-receptor-mediated inhibition of noradrenaline release from the rat stomach. Further examinations would be necessary to clarify the mechanism of endothelin ET_B receptor-mediated inhibition of noradrenaline release from the gastric sympathetic nerve terminals.

In conclusion, the present findings indicate that the activation of endothelin ET_A and ET_B receptors located on the gastric sympathetic nerve terminals inhibits the release of noradrenaline in rats; endothelin ET_A receptor-mediated inhibition is carried out by pertussis toxin-sensitive and indomethacin-insensitive mechanisms.

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