Contribution of Nitric Oxide to the Presynaptic Inhibition by Endothelin ET$_B$ Receptor of the Canine Stellate Ganglionic Transmission

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ABSTRACT

We previously reported that endothelin (ET) 3 inhibited presynaptically the dog stellate ganglionic transmission. Here, we report the investigation of the possible involvement of nitric oxide pathway in the endothelin-induced inhibition of the ganglionic transmission. The amount of acetylcholine released by preganglionic stimulation for 10 min was concentration-dependently inhibited after exposure to ET-3 (10$^{-9}$–10$^{-6}$ M) or IRL-1620, endothelin ET$_B$ receptor agonist (10$^{-8}$–10$^{-5}$ M). The inhibition was antagonized by pretreatment with a nonselective endothelin receptors antagonist (bosentan) and an ET$_B$ receptor antagonist (BQ-788) or a neuronal nitric oxide synthase inhibitor, 3-bromo-7-nitroindazole, but was not inhibited by a selective ET$_A$ receptor antagonist, BQ-123. The reduction induced by ET-3 was also antagonized by treatment with a selective inhibitor of soluble guanylyl cyclase, 8-bromo-cGMP analog, 8-bromo-3,5-cyclic monophosphate and nitric oxide donor, S-nitroso-N-acetylpenicillamine. Exposure to ET-3 or IRL-1620 for a 30-min period increased the levels of total nitric oxide (NO), nitrite plus nitrate NOx concentration in the incubation medium, with the increase in NOx also being antagonized by BQ-788 at the same concentration. The ET-3-induced increase in NOx was antagonized by treatment with the same concentration of 3-bromo-7-nitroindazole or a selective inhibitor of receptor-mediated Ca$^{2+}$ entry, 1-[b-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole (10$^{-5}$ M), and with a calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. These results indicate that ET$_B$ receptor activation inhibits the sympathetic ganglionic transmission via reducing acetylcholine release from presynaptic nerve terminals, although this inhibition also seems to involve the ET$_B$ receptor-operated Ca$^{2+}$-calmodulin-dependent activation of endogenous nitric oxide production.

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ABBREVIATIONS: ET, endothelin; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; IPHC, isopropylhomocholine chloride; SNAP, S-nitroso-N-acetylpenicillamine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 8-bromo-cGMP, 8-bromoguanosine-3,5-cyclic monophosphate and nitric oxide donor, S-nitroso-N-acetylpenicillamine. Exposure to ET-3 or IRL-1620 for a 30-min period increased the levels of total nitric oxide (NO), nitrite plus nitrate NOx concentration in the incubation medium, with the increase in NOx also being antagonized by BQ-788 at the same concentration. The ET-3-induced increase in NOx was antagonized by treatment with the same concentration of 3-bromo-7-nitroindazole or a selective inhibitor of receptor-mediated Ca$^{2+}$ entry, 1-[b-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole (10$^{-5}$ M), and with a calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. These results indicate that ET$_B$ receptor activation inhibits the sympathetic ganglionic transmission via reducing acetylcholine release from presynaptic nerve terminals, although this inhibition also seems to involve the ET$_B$ receptor-operated Ca$^{2+}$-calmodulin-dependent activation of endogenous nitric oxide production.
ganglionic transmission, while also specifying the ET receptor distribution of the NO system to the ET-induced inhibition of concept that ET may play a role in regulating the functions in 

ane A2 and NO (D’Orléans-Juste et al., 1994). In the whole sostigmine (10−6 M) placed in a dish containing Locke’s solution in the presence of phy-
the experiments of acetylcholine assay, the tissue specimens were 

late ganglia were removed, along with about 3 cm of the pregangli-
vard animal respirator (model 613; Harvard, Millis, MA). Both stel-
was cannulated, and artificial ventilation was maintained by a Har-

anesthetized with pentobarbital sodium (30 mg/kg i.v.). The trachea 

during that time they underwent general medical examination. Each 

put into the microtest tube containing 0.5 ml of Locke’s solution 

without physostigmine at 37°C and was then gassed with a mixture of 95% O2 and 5% CO2.

At the end of the experiment, the ganglion without nerve trunk was weighed after the removal of any excess moisture by pressing between filter paper.

**Determination of Acetylcholine.** Acetylcholine was measured by HPLC with electrochemical detection as described by Eva et al. (1984) and Potter et al. (1983). A standard mixture of acetylcholine, choline, and isopropylmethylcholine chloride at each concentration of 2 × 10−3 M was prepared daily from the stock solution of 2 × 10−3 M stored at 4°C. Isopropylmethylcholine chloride (internal standard, 15 μl of 2 × 10−5 M) was added into 300 μl of sample solution and filtered through a 0.22-μm membrane filter (UFC30GV00; Millipore, Tokyo, Japan). Aliquots of the standard and the filtered samples, 5 μl and 10 μl, were injected, respectively, into the HPLC system. The temperature of the enzyme column was maintained at about 33°C by a column heater (U-620; Sugai, Tokyo, Japan). The mobile phase consisted of 0.1 M disodium hydrogen phosphate buffered to pH 8.0 with phosphoric acid, containing 0.6 M tetrameth-
ylammonium chloride and 1.2 M sodium 1-decanesulfonate. The buffer was first prepared and filtered through a 0.45-μm membrane filter (Toyo Roshi, Tokyo, Japan). Tetramethylammonium chloride and sodium 1-decanesulfonate were added, and the solution was degassed by bubbling helium gas at a flow rate of 100 ml/min for 30 min. The pumping rate of the mobile phase was 1.0 ml/min.

**Acetylcholine Release.** In the experiments performed on the isolated ganglia, the output of acetylcholine was collected for a period of 10 min during preganglionic stimulation. The first and second samples were untreated, and thereafter every third sample was exposed to the drugs. Fresh medium containing the corresponding agents at the same concentration was introduced before the preincubation to remove the resting amount of acetylcholine released during the preincubation periods. Five to six samples were taken in the acetylcholine assay experiment.

Tissues were exposed to ET-3, IRL-1620, 8-bromoguanosine-3,5-
cyclic monophosphate (8-bromo-cGMP), S-nitroso-N-acetylpenicillia-
mine (SNAP), bosentan, BQ-123, BQ-788, 3-bromo-7-nitroindazole, and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) for 20 min as described (Ohjimi et al., 1994). The drug effects on acetylcholine release were evaluated as absolute values of the overflow evoked by the preganglionic stimulation. The content of acetylcholine released into the 0.7 ml incubation medium after the 10-min preganglionic stimulation period was calculated based on the absolute values of acetylcholine in a 10-μl aliquot of the sample solution detected by HPLC and was expressed as the percentage of initial acetylcholine output elicited by the first preganglionic stimulation.

**Measurement of NO2− and NO3− Levels.** The isolated ganglia, with their preganglionic trunk removed, were placed in a tube con-

Materials and Methods

**Animal Care.** Mongrel adult dogs of either sex, provided by the Fukuoka City Animal Control Center, were kept for about 1 week in the animal laboratory (Fukuoka University Animal Center), and during that time they underwent general medical examination. Each dog was housed in an individual cage in a temperature-controlled room (22°C) which was humidified (50–60%) and maintained on a 12-h/12-h light/dark cycle (8 AM/8 PM). All animals had free access to water and were fed standard solid laboratory food (ED-1, 300 g/dog/day; Sanwa Chemicals Inc., Tokyo, Japan). Only animals in good physical health were used in the experiments. The experimental procedures were carried out under the protocols approved by the Animal Care Committee of Fukuoka University and in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**In Vitro Experiments on the Ganglia.** All dogs (6–12 kg) were anesthetized with pentobarbital sodium (30 mg/kg i.v.). The trachea was cannulated, and artificial ventilation was maintained by a Har-

ard animal respirator (model 613; Harvard, Millis, MA). Both stel-
late ganglia were removed, along with about 3 cm of the pregangli-

neonic sympathetic nerve for the nerve stimulation experiments. For 

the experiments of acetylcholine assay, the tissue specimens were placed in a dish containing Locke’s solution in the presence of phy-

sostigmine (10−6 M) at room temperature and gassed with a mixture of 95% O2 and 5% CO2, and the tissue sheath around the ganglia was carefully removed. The experimental procedures were principally performed according to the methods described by Ohjimi et al. (1994). The composition of the Locke’s solution was 136 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 1.2 mM MgCl2, 1.2 mM NaH2PO4, 20.0 mM NaHCO3, and 11.0 mM glucose.

The ganglion was transferred to a microtest tube containing 0.7 ml of fresh medium (Locke’s solution containing physostigmine, 10−6 M) at 37°C, gassed with a mixture of 95% O2 and 5% CO2, and equilibrated for 60 min. The preganglionic nerve was laid across a bipolar platinum electrode suspended just above the surface of the solution and stimulated by square wave pulses of 1-ms duration at a fre-

quency of 5 Hz on 60 V strength for 10 min each with an electric stimulator (SEN-3201; Nihon Kohden, Tokyo, Japan) delivered via an isolation transformer (SS-2015 m; Nihon Kohden). The interval between each successive preganglionic stimulation was 10 min.

In the NO determination experiments, both sides of the stellate ganglia without preganglionic nerves were prepared under the same experimental conditions as those described above. The ganglion was put into the microtest tube containing 0.5 ml of Locke’s solution without physostigmine at 37°C and was then gassed with a mixture of 95% O2 and 5% CO2.

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taining 0.5 ml of Locke’s solution and were oxygenated with 95% O2/5% CO2 and incubated at 37°C in a water bath. The output of NO was collected over periods of 30-min incubation. The first through third samples were untreated, and thereafter fourth through sixth samples were exposed to the drugs. The intervals between each successive incubation period was 20 min. The agents were administered after three consecutive untreated samples had been collected as the basal levels. The left ganglia were used for the control groups, and the right for the antagonist-treated groups. Fresh medium containing the corresponding antagonists at the same concentration was introduced after the preincubation to remove the amount of NO released during the preincubation periods of antagonists.

Tissues were exposed to agonists (ET-3, IRL-1620, SNAP, and 8-bromo-cGMP) for 30 min, and antagonists (BQ-123, BQ-788, 3-bromo-7-nitroindazole, ODQ, SKF-96365, and W-7) for 20 min. After drug incubation, an aliquot was removed and put immediately into chilled acetone with dry ice, then stored at below -40°C until the assay. Because a relatively high amount of NO can diffuse into the incubation medium which is more distant than neuronal cells in close proximity separated by the synaptic cleft, higher concentrations of ET analog than those used for affecting the release of acetlycholine are needed to detect NO metabolites in the medium in the current experiment. The NO metabolites content released into 0.5 ml of the medium after 30 min incubation period was calculated from the absolute values of the NO metabolites in a 20-μl aliquot of the sample solution detected by the NO detector-HPLC system (ENO-20; Eicom, Kyoto, Japan), and was expressed as the percentage of the initial NO metabolites output elicited by a 30-min incubation in untreated ganglia. NO x and NO2 were separated by a reversed-phase separation column packed with poly styrene polymer (NO-PAR, 4.6 × 50 mm; Eicom, Kyoto, Japan), and NO2 was reduced to NO2 in a reduction column packed with copper-plated cadmium filings (NO-RED; Eicom). NO2 was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven heated to 35°C. The absorbance of the color of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, composed by Eicom, consisted of 10% methanol containing 0.15 M NaCl-NH4Cl and 0.5 g/liter of EDTA4Na4 and was delivered by a pump at a rate of 0.33 ml/min. The Griess reagent, composed by Eicom (1.25% HCl containing 5 g/liter of sulfanilamide with 0.25 g/liter N-naphthylethlyenediamine), was delivered at a rate of 0.1 ml/min. The accurate levels of NO2 and NO2 were calculated by subtracting the contamination of NO2 in Locke’s solution from the sample values in each experiment. The total NO metabolite level (NOx) is the sum of the NO2 and NO2 levels.

When calcium dependence of ET-3-induced increase of NOx levels was examined, CaCl2 was omitted from Locke’s solution and was replaced with 2 mM EGTA (Ca2+-free Locke’s solution).

Drugs Used. The drugs used were: ET-3 and Suc-Glu6,Ala11,15-ET-1(8–21) (IRL-1620) (Peptide Institute Inc., Osaka, Japan); N-cis-2,6-dimethyl-piperidinocarbonyl-i-g-methyl-Leu-i-1-methoxy carbonyl-Trp-n-Nle (BQ-788) (Peninsula Laboratories Inc., Belmont, CA); 3-bromo-7-nitroindazole, ODQ, and SNAP (Tocris Cookson Ltd., Bristol, UK); 1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl]-1H-imidazol (SKF-96365; Biomol Research Laboratories Inc., Plymouth Meeting, PA); ethyleneglycol bis(2-aminoethyl ether) tetraacetatic acid (EGTA-2Na; Nacalai Tesque Inc., Kyoto, Japan); N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), physostigmine hemisulfate and 8-bromoguanosine-3,5-cyclic monophosphate (8-bromo-cGMP; Sigma Chemical Co., St. Louis, MO); Cyclo(t-a-aspartyl-l-prolyl-b-valyl-l-leucyl-d-tryptophyl) (BQ-123), and 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)-2,2’-bipyrimidin-4-yl] benzene sulphonamide sodium salt (bosentan) were a generous gift from Banyu Pharmaceutical Co. Ltd. (Tsukuba, Japan) and Hoffmann-La Roche Ltd. (Basel, Switzerland), respectively.

Statistical Analysis. Each value represents the mean ± S.E. The values between more than two mean values of the dose-response run in the same ganglia were evaluated by the Dunnett test. Comparisons of the left-untreated with antagonists and right-treated ganglia in the same animals were performed using the ANOVA, and followed by the Bonferroni t test. Values of p < .05 were considered to be significantly different.

Results

Inhibitory Effects of ET Agonists on Acetylcholine Release. When the preganglionic nerve was stimulated, the amount of acetylcholine release was increased in a frequency-dependent manner, being one-half the maximum at 5 Hz and maximal at 20 Hz (Ohjimi et al., 1994). According to this finding, the preganglionic nerve stimulation was applied at 5 Hz for the following experiments. As shown in Fig. 1, the output of acetylcholine released by preganglionic stimulation for each 10-min period was reduced in a dose-dependent fashion in the presence of ET-3 at concentrations of 10–9 to 10–6 M or IRL-1620 at 10–9 to 10–6 M; the release was reduced to 60.9% and 66.6% of the control at maximal concentrations of 10–6 and 10–5 M, respectively.

Effects of ET Antagonists on ET-3-Induced Reduction of Acetylcholine Release. As shown in Fig. 2, the ET-3-induced inhibition of acetylcholine release during preganglionic stellate stimulation was clearly antagonized by incubation with a nonselective ETA and ETB receptors antagonist, bosentan, or an ETB receptor antagonist, BQ-788, each at a concentration of 10–6 M, whereas the inhibition was not affected by an ETA receptor antagonist, BQ-123, at 10–6 M. The acetylcholine release was not affected significantly by the application of either drug alone in the absence of ET-3. The selective ETA receptor antagonist, BQ-123, competitively inhibits the effect of ET-1 in the human umbilical artery (pA2, 6.9), and the contraction induced by ET-3 in the vein is inhibited by BQ-788 (pA2, 7.6) (Bogoni et al., 1996). Therefore, the concentration of BQ-123 and BQ-788 used in the present study, 10–6 M, is sufficient to antagonize the...
Effects induced by ET<sub>A</sub> and ET<sub>B</sub> receptor activation, respectively.

Effects of Neuronal NOS (nNOS) Inhibitor on ET Agonist-Induced Reduction of Acetylcholine Release.
As shown in Fig. 3, the ET-3- and IRL-1620-induced inhibitions of acetylcholine release during preganglionic stellate stimulation were antagonized by incubation with 10<sup>-5</sup> M 3-bromo-7-nitroindazole. The application of 3-bromo-7-nitroindazole alone in the absence of ET-3 and IRL-1620 did not affect the acetylcholine release significantly (Fig. 3). Long-term potentiation in the rat dentate gyrus is inhibited by the inhibitory response of NOS activity to 3-bromo-7-nitroindazole at dose of 3 × 10<sup>-5</sup> M (Wu et al., 1997).

In the preliminary experiment, the calmodulin antagonist, W-7, at a dose of 10<sup>-5</sup> M, without affecting acetylcholine release during preganglionic stimulation, also antagonized the ET-3-induced inhibition of acetylcholine release (data not shown).

Inhibitory Effects of 8-Bromo-cGMP and S-Nitroso-N-Acetylpenicillamine on Acetylcholine Output. As shown in Fig. 4, 8-bromo-cGMP, a membrane-permeable cGMP analog, and S-nitroso-N-acetylpenicillamine, a NO donor, at concentrations of 10<sup>-8</sup> to 10<sup>-6</sup> M and 10<sup>-6</sup> to 10<sup>-3</sup> M, respectively, decreased in a dose-dependent manner the acetylcholine output elicited by preganglionic stimulation. The reduction of the acetylcholine output at a maximum concentration of 10<sup>-5</sup> M by 8-bromo-cGMP was 42.9% and of 10<sup>-3</sup> M by S-nitroso-N-acetylpenicillamine 42.5%, respectively.

Effects of ODQ on the ET-3-Induced Reduction of Acetylcholine Release. As shown in Fig. 5, the reduction of acetylcholine output induced by ET-3 at concentrations of 10<sup>-9</sup> to 10<sup>-6</sup> M was antagonized by incubation with a soluble guanylyl cyclase inhibitor, ODQ (10<sup>-4</sup> M). The agent, per se, at the concentration used did not influence the acetylcholine output elicited by preganglionic stimulation. ODQ at a dose of 10<sup>-5</sup> M abolishes an elevation of cGMP accumulation

![Fig. 2. Antagonism by a nonselective endothelin ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist, bosentan, and selective ET<sub>B</sub> receptor antagonist, BQ-788, of the ET-3-induced reduction in acetylcholine output by preganglionic stimulation in untreated (■) and those in the presence of 10<sup>-6</sup> M bosentan (■) or 10<sup>-6</sup> M BQ-788 (■) or BQ-123 (■). In six groups of ganglia, the control mean basal amount of acetylcholine release before preganglionic stimulation was 8.9 ± 1.27, 10.3 ± 2.03, 9.3 ± 1.76, 6.8 ± 0.83, 5.8 ± 1.18, and 6.1 ± 1.08 nmol/10 min/g, respectively. Each column represents the mean of five ganglia. *p<.05. †, significant difference between the values before and after ET-3 or the same ganglion (p<.05). Each column represents the mean of five ganglia.

![Fig. 3. Antagonism by a nNOS inhibitor, 3-bromo-7-nitroindazole, of the ET-3- or IRL-1620-induced reduction in acetylcholine output elicited by preganglionic stimulation in untreated (□) and 3-bromo-7-nitroindazole (10<sup>-5</sup> M)-treated (■). In four groups of ganglia, the mean basal amount of acetylcholine release before drug was 6.1 ± 1.14, 7.3 ± 0.67, 6.5 ± 1.01 and 7.1 ± 1.09 nmol/10 min/g, respectively. *p<.05. †, significant difference between the values before and after ET-3 or IRL-1620 in the same ganglion (p<.05). Each column represents the mean of five ganglia.

![Fig. 4. Concentration-response curves for inhibition by a membrane-permeable cGMP, 8-bromo-cGMP, and a NO donor, SNAP, of acetylcholine output elicited by preganglionic stimulation in 8-bromo-cGMP (■) and SNAP (○)-treated ganglia. In two groups of ganglia, the mean basal amount of acetylcholine release before drug was 8.5 ± 1.96 and 6.5 ± 0.78 nmol/10 min/g, respectively. *p<.05. †, significant difference between the values before and after the agents in the same ganglion (p<.05). Each point represents the mean of five ganglia. See the legend to Fig. 1 for an additional explanation.](image-url)
Effects of ET-3 and IRL-1620 on NO\(_x\) Levels. As shown in Fig. 7, the ET-3- and IRL-1620-induced increases in the NO\(_x\) levels were completely antagonized by ET\(_4\) receptor antagonist, BQ-788, at a concentration of 10\(^{-5}\) M, which did not affect the basal NO\(_x\) levels in the absence of either ET-3 or IRL-1620.

Effects of SKF-96365 or the Removal of Extracellular Ca\(^{2+}\) on ET-3-Induced NO\(_x\) Levels. The ET-3-induced increase in the NO\(_x\) levels was not only antagonized by a selective receptor-mediated Ca\(^{2+}\)-entry inhibitor, SKF-96365 (10\(^{-5}\) M) (Fig. 8) but also lowered the extracellular Ca\(^{2+}\) levels by incubating Ca\(^{2+}\)-free Locke’s solution (data not shown). SKF-96365 at a dose of 3 \(\times\) 10\(^{-5}\) M eliminates ET agonist-mediated [Ca\(^{2+}\)]\(i\) increase in bovine corneal epithelial (Tao et al., 1997).

Effects of 8-Bromo-7-Nitroindazole and W-7 on ET-3-Induced NO\(_x\) Levels. As shown in Fig. 9, the ET-3-induced increase in the NO\(_x\) levels was antagonized by a nNOS inhibitor, 8-bromo-7-nitroindazole, at 10\(^{-5}\) M or a calmodulin antagonist, W-7, at 10\(^{-4}\) M. These agents, per se, at the concentrations used did not influence the basal NO\(_x\) levels in the absence of ET-3. W-7 at doses of 5 \(\times\) 10\(^{-7}\) to 5 \(\times\) 10\(^{-4}\) M dose-dependently inhibited NO\(_x\) synthesis stimulated by ET-3 in cultured bovine endothelial cells (Hirata and Emori, 1993).

**Discussion**

In the current study, the contribution of ET receptor subtype specificity and the NO system to the presynaptic inhibition induced by ET agonists of the dog stellate ganglionic transmission were investigated. According to the experiment using the acetylcholine assay as described previously (Ohjimi et al., 1994; Kushiku et al., 1995), there were no changes in the elicited output of acetylcholine during eight successive preganglionic stimulations with 10-min intervals. Under such experimental conditions, the output of acetylcholine elicited by the preganglionic stimulation was decreased by exposure of the isolated stellate ganglia to ET-3 at 10\(^{-9}\) to 10\(^{-8}\) M (Kushiku et al., 1995). In the present study, ET-3 at the same concentrations in as those used in our previous report (Kushiku et al., 1995), and ET\(_4\) receptor agonist, IRL-1620, at 10\(^{-8}\) to 10\(^{-5}\) M also inhibited the output of acetyl-
choline from the isolated stellate ganglia. Therefore, the efficacy of IRL-1620 was lower than that of ET-3.

ET has also been suggested to exert an inhibitory action on the neurotransmission of the preganglionic and postganglionic nerve terminals. ET inhibits the nerve stimulation-induced contractile responses and fractional release of [3H]acetylcholine from the guinea pig ileum as well as [3H]norepinephrine from the guinea pig femoral artery (Wiklund et al., 1988; Wiklund et al., 1989). ET-3 also inhibits the sympathetic ganglionic transmission by reducing the acetylcholine release at preganglionic terminals (Kushiku et al., 1995). However, the specificity of the receptor subtype for the ET-induced inhibition of the sympathetic neurotransmission has yet to be determined. In the present study, the nonspecific ET \(_A\) and ET \(_B\) receptor antagonist, bosentan, and the specific ET \(_B\) receptor antagonist, BQ-788, but not the ET \(_A\) receptor antagonist, BQ-123, completely antagonized the ET-3- and IRL-1620-induced inhibition of acetylcholine release elicited by preganglionic stellate stimulation. These results indicate the inhibition induced by ET analog to be mediated by the ET \(_B\) receptor activation at the ganglion.

NOS-immunoreactivity is distributed in the preganglionic sympathetic neurons of rat pre- and paravertebral (Anderson et al., 1993; Blottner and Baumgarten, 1992) and superior cervical (Okamura et al., 1995) ganglion. In contrast, NOS is also localized in a population of sympathetic postganglionic neurons in the guinea pig paravertebral and inferior mesenteric ganglia (Höhler et al., 1995) and in the cat stellate and lower lumbar ganglia (Anderson et al., 1995). Furthermore, both nNOS and endothelial NOS are constitutively expressed in neuronal tissue (Dinerman et al., 1994). Hypoxia increases the nNOS mRNA expression in the nodose ganglion and cerebellum, respectively, whereas it had no significant effect on the endothelial NOS levels (Prabhakar et al., 1996). Therefore, these results indicate that nNOS is present in the pre- and/or postganglionic sympathetic neurons.

In the rabbit perfused kidney, the increase of perfusion pressure induced by ET-1 was potentiated by BQ-788, a selective ET \(_B\) receptor antagonist and by N\(^{-}\)-nitro-l-arginine methyl ester, a NOS inhibitor (D’Orléans-Juste et al., 1994). The inhibition of NOS with N\(^{-}\)-monomethyl-l-arginine completely abolished the renal vasodilation induced by ET-1 and ET-3 and ET-3-displayed diuresis and natriuresis in the dogs (Chou and Porush, 1995). Furthermore, in the whole rat adrenal medulla, ET-1 and ET-3 stimulate NO-induced cGMP generation through ET \(_B\)-receptor activation (Mathison and Israel, 1998). In the present experiment on the sympathetic ganglia, the preganglionic inhibition by ET-3 or ET \(_B\) receptor agonist, IRL-1620, of the acetylcholine output elicited by preganglionic stimulation was also inhibited by the treatment with 3-bromo-7-nitroindazole, a selective nNOS inhibitor. On the other hand, the exposure of these ET analogs increased the levels of total NO metabolites in the incubation medium, and this increase disappeared after treatment with BQ-788 and 3-bromo-7-nitroindazole. Furthermore, weak efficacy of IRL-1620 than ET-3 on the release of NO metabolites is well correlated with that on affecting the acetylcholine release in the present study. These facts suggest that the increase of NO formation mediated by ET \(_B\) receptor activation may participate in the ET-induced preganglionic inhibition in the canine stellate ganglion.

Endogenous NO is produced from L-arginine by NOS, thus resulting in the stoichiometric production of L-citrulline (Mayer et al., 1989; Palmer and Moncada, 1989). The constitutive NOS exists in endothelial cells (Mayer et al., 1989; Busse and Mülsch, 1990) and neuronal cells (Bredt et al., 1990), which is Ca\(^{2+}\)-calmodulin- and NADPH-dependent (Palmer and Moncada, 1989). NO identified in many tissues affects a soluble guanylyl cyclase (Arnold et al., 1977; Murad et al., 1978), and binds tightly to the heme region of cyclase and causes an increase in the cGMP levels, which thus affects the ion channel (Rascón et al., 1992). In the current study, the preganglionic inhibition by ET agonists of acetylcholine release elicited by preganglionic stellate stimulation was inhibited by treatment with ODQ, a selective inhibitor of soluble guanylyl cyclase, and this inhibition was mimicked by SNAP, an NO donor, and 8-bromo-cGMP, a membrane-permeable cGMP. In addition, ET agonists stimulated the pro-
duction of NO$\text{\textsuperscript{2-}}$, and this accumulation was inhibited by W-7, a calmodulin antagonist, SKF-96365, a selective inhibitor of receptor-mediated Ca$^{2+}$ entry and incubating Ca$^{2+}$-free medium. SKF-96365 inhibits a receptor-mediated Ca$^{2+}$ entry as well as voltage-gated Ca$^{2+}$ entry without affecting the internal Ca$^{2+}$ release at the concentrations used in the present experiment, which were the same as that used in a previous report (Merritt, 1990). The results indicate that ET agonists stimulate the receptor-mediated Ca$^{2+}$-dependent formation of NO metabolites activating the soluble guanylyl cyclase, thus leading to an increased cGMP content in the dog stellate ganglia.

In conclusion, ET inhibits sympathetic ganglionic transmission at presynaptic sites via ET$\text{\textsubscript{B}}$ receptor by reducing the output of acetylcholine release through the stimulation of the NO production and cGMP pathways.

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