Endothelin-1 increases the neuronal activity and augments the responses to glutamate in the NTS

MIWAKO SHIHARA,1 YOSHITAKA HIROOKA,1 NOBUAKI HORI,2 ISAMU MATSUO,1 TATSUYA TAGAWA,1 SATOSHI SUZUKI,1 NORIO AKAIKE,3 AND AKIRA TAKESHITA1

1Research Institute of Angiocardiology and Cardiovascular Clinic; 2Department of Physiology, Kyushu University School of Medicine; and 3Department of Pharmacology, Kyushu University School of Dentistry, Fukuoka 812–8582, Japan

Shihara, Miwako, Yoshitaka Hirooka, Nobuaki Hori, Isamu Matsuo, Tatsuya Tagawa, Satoshi Suzuki, Norio Akaike, and Akira Takeshita. Endothelin-1 increases the neuronal activity and augments the responses to glutamate in the NTS. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R658–R665, 1998.—The aims of this study were to determine 1) whether endothelin (ET)-1 affects the neuronal activity of the NTS neurons, 2) whether specific ET receptor antagonists affect the neuronal activity of the NTS neurons, and 3) whether ET-1 or ET receptor antagonists modulate the responses of the nucleus of the solitary tract (NTS) neurons to L-glutamate (Glu). The single-unit discharge was extracellularly recorded with a fine electrode from medulla brain slice preparations of rats. ET-1 and Glu were iontophoretically applied to the recorded neuron. Both ET-1 and Glu increased the neuronal activity. The ETα receptor antagonist BQ-123 attenuated the basal neuronal activity. ET-1 augmented the magnitude of the increases in the neuronal activity evoked by Glu, and these responses were antagonized by BQ-123. These studies suggest the following conclusions: 1) ET-1 increases the neuronal activity of the NTS neurons via ETα receptors, 2) endogenous ET plays a controlling role of the neuronal activity of NTS neurons, and 3) ET-1 augments the responses evoked by Glu, believed to be the neurotransmitter from the solitary tract, via ETα receptors. These results suggest that ET-1 facilitates synaptic transmission in the NTS.

brain stem; baroreflex; cardiovascular system

THERE IS CONSIDERABLE evidence that endothelin (ET) may play an important function as a neuromodulator and/or a neurotransmitter within the central nervous system (19, 24). In addition, various studies using immunohistochemistry, autoradiography, and in situ hybridization techniques have demonstrated the existence of all components of the ET system, including ET itself, its receptors, and ET-converting enzyme activity, in the brain (13, 15, 18–20, 28, 29). ET binding sites are found in the hypothalamus and the brain stem, which are known to be important sites for cardiovascular regulation (4, 16). In the brain stem, ET-1 and ET-1 binding sites are abundant in the nucleus of the solitary tract (NTS) and the ventrolateral medulla (13, 15, 18, 19). These results suggest that ET-1 within the brain stem, including the NTS, may contribute to the control of sympathetic nerve activity and arterial pressure.

The NTS is the site where afferent fibers from arterial baroreceptors, chemoreceptors, cardiopulmonary receptors, and other visceral receptors make their first synapse (1, 4, 5, 16). The NTS is thus believed to play an important role in the integration of the autonomic control of the cardiovascular system (1, 4, 5). Microinjection of ET-1 into the NTS is known to decrease both arterial pressure and heart rate in vivo (7, 24), thus suggesting that ET-1 increases neuronal activity of NTS neurons. However, it is not known whether ET-1 directly affects neuronal activity because microinjection of ET-1 into the NTS may cause medullary ischemia, which results in changes in the arterial pressure (6, 21, 22, 25). In addition, the role of endogenous ET within the NTS is yet to be elucidated. Furthermore, it has also been suggested that the injection of ET-1 into the cisterna magna sensitizes the arterial baroreflex control of heart rate (12). It is thus possible that ET-1 within the NTS influences the effect of such major neurotransmitters as L-glutamate (Glu) (32) or the responses of the NTS neurons to it.

To investigate the role of ET-1 in these pathways, we cut the semihorizontal brain slices containing the NTS of rats and recorded extracellular single-unit discharges from the medial NTS (mNTS) that responded to solitary tract (ST) stimulation, and we attempted to determine 1) whether ET-1 affects the neuronal activity of the NTS neurons, 2) whether selective ET receptor antagonists affect the neuronal activity of the NTS neurons, and 3) whether ET-1 modulates the excitability of the NTS neurons evoked by Glu.

A preliminary report of these data has been presented (26).

METHODS

Slice preparation. Wistar-Kyoto rats (WKY: 100–150 g, 4–6 wk old) were used in all experiments. Under ether anesthesia, the rat was euthanized by cervical dislocation, and the brain stem and cerebellum were rapidly removed to cold Krebs-Ringer solution containing (in mM) 126 NaCl, 5 KCl, 2.4 CaCl2, 1.3 MgSO4, 1.26 KH2PO4, 26 NaHCO3, and 10 glucose, saturated with 95% O2 and 5% CO2. A semihorizontal
Effects of exogenous ET-1 or endogenous ET on the responses to iontophoretically applied Glu. To determine whether ET-1 modulates the responses to Glu, a major neurotransmitter in the NTS, we iontophoretically applied Glu before and after iontophoretic ET-1 application (n = 21). Glu was iontophoretically applied for 1 s at a fixed interval of 1 min. After the basal spontaneous neuronal activity and the stable responses to Glu were confirmed, ET-1 was iontophoretically applied for 5 s. Glu application every minute was continued.

To determine whether the responses to Glu are influenced by endogenous ET, we applied Glu iontophoretically during the perfusion with BQ-123 or BQ-788. After confirming the basal spontaneous neuronal activity and the stable responses to iontophoretically applied Glu, Krebs-Ringer solution containing BQ-123 (10^-4 M, n = 10) or BQ-788 (10^-4 M, n = 10) was perfused for 7 min to the recording chamber at a flow rate of 3 ml/min.

To investigate whether the blocking effects of BQ-123 were specific, we examined whether BQ-123 (10^-4 M) modulates the effect of ACh (0.5 M in distilled water, pH 3.5) (11, 27) on the neuronal activity of the NTS. ACh was applied iontophoretically.

Histological studies. At the end of the experiments, the slices were fixed with a 4% paraformaldehyde solution in PBS, frozen, sectioned at 100-µm thickness, and then stained with neutral red. A representative slice preparation is shown in Fig. 1.

Drugs. The following drugs were used: Glu (Sigma Chemical, St. Louis, MO), ET-1 (Peptide Institute, Osaka, Japan), ACh (Torris Cookson, St. Louis, MO), ET_\beta receptor antagonist BQ-788 (Novabiochem, Tokyo, Japan) ET_\alpha receptor antagonist BQ-123 was a gift from Banyu Pharmaceutical, Tokyo, Japan.

Statistical analysis. The data are expressed as means ± SE. Statistical significance was evaluated by an analysis of variance with repeated measures or Student's paired t-test as appropriate. Differences were considered significant at a value of P < 0.05.

RESULTS

Response of the mNTS neurons to ET-1. Fifty-five neurons that responded to the ST stimulation were examined, and all of those neurons responded to iontophoretically applied Glu and ET-1. ET-1 increased the neuronal activity from 2.1 ± 0.2 to 5.4 ± 0.4 spikes/s (P < 0.01). These effects were transient and current dependent (Fig. 2) and were observed even during perfusion with a solution containing low Ca\(^{2+}\) and high Mg\(^{2+}\), which eliminated synaptic transmission (data not shown). Figure 2 shows that spikes decrease or cease after intense excitation by Glu and the other excitants. This may come from inactivation of sodium channels owing to strong depolarization or from activation of an electrogenic sodium pump, because these phenomena were even observed under low-Ca\(^{2+}\) and high-Mg\(^{2+}\) solution. The current itself (through one of the multibarrel pipettes filled with NaCl solution) did not affect the neuronal activity.

The increased neuronal activity evoked by iontophoretically applied ET-1 was attenuated by BQ-123 but not by BQ-788. The magnitude of increase in neuronal activity evoked by iontophoretically applied ET-1 was attenuated from 3.2 ± 0.6 to 2.2 ± 0.4 spikes/s during perfusion with BQ-123 (10^-3 M) (P < 0.05, n = 10).
magnitude of increase in neuronal activity evoked by iontophoretically applied ET-1 was attenuated from 2.3 ± 0.2 to 0.9 ± 0.2 spikes/s during perfusion with BQ-123 (10^{-4} M) (P < 0.01, n = 10, Fig. 3, A and C). The magnitude of increase in neuronal activity evoked by iontophoretically applied ET-1 did not differ before or during perfusion with a high concentration of BQ-788 (10^{-4} M) (2.9 ± 1.0 vs. 2.6 ± 0.7 spikes/s, NS, n = 10, Fig. 3, B and C).

To examine whether the blocking effect of BQ-123 on the excitatory response evoked by ET-1 was specific for ET-1, we applied ACh iontophoretically to the neurons in the mNTS during perfusion with BQ-123 (10^{-4} M). The magnitude of increase in neuronal activity evoked by the iontophoretically applied ACh did not change before or during perfusion with BQ-123 (2.6 ± 0.9 vs. 3.4 ± 0.7 spikes/s, NS, n = 3).

Effects of ET receptor antagonists on the spontaneous activity of the mNTS neurons. Perfusion with BQ-123 (10^{-4} M) decreased the control spontaneous neuronal activity from 2.2 ± 0.2 to 1.8 ± 0.2 spikes/s (P < 0.01, n = 23), but that with BQ-788 (10^{-4} M) did not alter the basal spontaneous neuronal activity (from 3.0 ± 0.5 to 3.0 ± 0.5 spikes/s, NS, n = 13).

Effects of ET-1 on the response to iontophoretically applied Glu. Iontophoretically applied Glu to the same site as ET-1 increased the neuronal activity of the mNTS neurons as shown in Fig. 4A. After ET-1 application, the magnitude of increase in neuronal activity evoked by iontophoretically applied Glu was augmented from 4.6 ± 0.5 to 7.4 ± 0.8 spikes/s (P < 0.01, n = 21, Fig. 4B). This augmentation peaked at 1–5 min after ET-1 application and then gradually returned to the control response by ~20 min (Fig. 4A).

Fig. 1. Experimental preparation of the brain stem slice. A: photomicrograph of a 100-µm section cut with neutral red staining. NTS, nucleus of the solitary tract; AP, area postrema; DMV, dorsal motor nucleus of the vagus; ST, solitary tract; 4th, fourth ventricle. B: schematic drawing of slice preparation. Rec, recording electrode; IP, iontophoretic electrode; Stim, stimulating electrode.

Fig. 2. Example of increased neuronal activity evoked by iontophoretically applied endothelin (ET)-1 (10^{-4} M; 10, 20, and 40 nA) on a single neuron of the medial NTS (mNTS) in a rat brain stem slice. Raw neurograms are shown at top, and firing rate is shown at bottom. Iontophoretically applied ET-1 increased neuronal activity as a function of applied current.
Effects of ET receptor antagonists on the responses to iontophoretically applied Glu. The magnitude of increase in neuronal activity evoked by iontophoretically applied Glu was attenuated from 4.5 ± 0.3 spikes/s to 2.9 ± 0.6 spikes/s during perfusion with BQ-123 (10⁻⁴ M) (A) or BQ-788 (10⁻⁴ M) (B) are shown. C and D: bar graphs showing change in the neuronal activity evoked by iontophoretically applied ET-1 before and during perfusion with BQ-123 (10⁻⁴ M) (C) or BQ-788 (10⁻⁴ M) (D). **P < 0.01 vs. values before ET-1.

DISCUSSION

Our results indicate that ET-1 increases the neuronal activity of mNTS neurons via ET₄ receptors. Second, these findings suggest that endogenous ET contributes to the control of the basal spontaneous neuronal activity of the mNTS neurons. Finally, ET-1 augmented the responses to Glu, which is considered to be the major neurotransmitter from baroreceptors to the NTS (32), via ET₄ receptors, thus suggesting that ET-1 may facilitate synaptic transmission in the NTS.

Effect of exogenously applied ET-1 on the neuronal activity of the mNTS neurons. We demonstrated that the iontophoretically applied ET-1 increased the neuronal activity of mNTS neurons. This finding also supports the results reported by other investigators (7, 24), which indicated that the microinjection of ET-1 into the NTS decreases both arterial pressure and heart rate in anesthetized rats in vivo. Furthermore, in the present study, iontophoretically applied ET-1 increased the neuronal activity of mNTS neurons during perfusion with modified Ringer solution containing low Ca²⁺ and high Mg²⁺, thus suggesting that ET-1 mainly acts on the postsynaptic membrane of neurons of the mNTS because the magnitude of increase in neuronal activity evoked by ET-1 after blocking synaptic transmission did not differ from that during perfusion with normal Ringer solution.

Because ET-1 is a potent vasoconstricotor agent, it is difficult to confirm in vivo whether the cardiovascular response of ET-1 results from neuronal activity or from the ischemia produced by intense vasoconstriction. It has been reported that ET-1 applied to the middle cerebral artery of the rat reduces the local cerebral blood flow (25). It has also been shown that the intracisternal administration of 5–500 pmol ET induces basilar artery contraction in vivo (22). Furthermore, it has been demonstrated that the intracisternal administration of 30 pmol ET-1 in WKY markedly increases the blood pressure with concomitant reductions in the blood flow through the caudal medulla (21). Our present studies using medulla slice preparations have clearly demonstrated that ET-1 affects neuronal activity of the mNTS directly. The possible increase of neuronal activity via ischemic insult by ET-1 injection in vivo systems can be excluded because the brain slice preparation is independent from the vascular system.

The increased neuronal activity evoked by ET-1 is mediated by ET₄ receptors because it was attenuated by BQ-123 but not by BQ-788. The effect is specific for
ET-1 because the responses to ACh did not change after BQ-123.

The role of endogenous ET-1 on the neuronal activity in the mNTS. Both ETA and ETB receptors are widely distributed in a variety of tissues, including the central nervous system. In the brain stem, there are abundant ET receptor binding sites. There are ETA receptor binding sites in the intermediate and the caudal NTS (15, 18, 29). The ETA receptor antagonist inhibited not only the responses to ET-1 but also the control spontaneous neuronal activity. These results thus indicate that responses to ET-1 are mediated by the ETA receptors located on the postsynaptic membrane and that endogenous ET helps control the spontaneous neuronal activity of the mNTS. It has been reported that microinjection of BQ-123 (24) may increase the spontaneous neuronal activity of NTS neurons, which thus influences basal arterial pressure and sympathetic nerve activity. The biphasic depressor/pressor changes in arterial pressure after microinjection of BQ-123 (24) probably indicate that this compound is a partial agonist and has a brief excitatory effect on the cell before blocking the receptors.

Augmented responses to Glu evoked by ET-1. Our findings do not clarify the precise mechanism(s) by which ET-1 augments the responses to Glu. In vascular smooth muscle, ET-1 elicits a biphasic increase in intracellular calcium concentration ([Ca^{2+}]_i) (10, 31). It has been shown that ET-1 induces the depolarization of the ventral root in the newborn rat spinal cord through the L-type calcium channel (33). It has also been shown that ET-1 modulates [Ca^{2+}]_i of neurons in primary cultures of rat cerebellum or slices of the hippocampus (14, 30). We thus speculate that ET-1 may increase [Ca^{2+}]_i by augmenting the release from the intracellular stores or by facilitating the influx through voltage-dependent or receptor-operated calcium channels in the neurons of the mNTS, which may augment the
response to Glu. Furthermore, D’Amico et al. (3) have shown that pretreatment with MK-801, an N-methyl-D-aspartate (NMDA) receptor antagonist, but not 6-cyano-7-nitroquinoxaline-2,3-dione, a non-NMDA receptor antagonist, blocks the cardiovascular responses caused by ET-1 in the periaqueductal gray area. Hashim et al. (8) have also shown that the hypotensive effect of intracerebroventricular ET-1 is attenuated by excitatory amino acid receptor antagonists. As a result, there may be some linkage between ET receptor activation and glutamate receptor activation, which may be related to an increase in \([\text{Ca}^{2+}]_i\).

We demonstrated that during perfusion with BQ-123, the neuronal responses to Glu were attenuated, thus suggesting that endogenous ET-1 may also influence synaptic transmission in the NTS. The effects of BQ-123 are specific because 1) the equimolar concentrations of ETB receptor antagonist did not affect the increased neuronal activity evoked by ET-1 in the mNTS and 2) BQ-123 did not affect the responses to iontophoretically applied ACh. The results of the present study suggest that ET-1 may augment synaptic transmission and may thus facilitate the arterial baroreflex. In that study, however, ET-1 caused marked hypotension and bradycardia. It is thus difficult to compare the reflex response because of the marked baseline differences in both the arterial pressure and heart rate before and after ET-1. In addition, intracisternal ET-1 also affects other important ET-1-sensitive areas, such as the ventrolateral medulla, more than the NTS (19). Furthermore, we could not determine from our study whether the neurons whose activity was augmented by Glu received inputs only from the arterial baroreceptors. It is possible that they received inputs from chemoreceptors and/or other visceral receptors (29). Further studies are needed to clarify the role of ET-1 on the neurons of the NTS that synapse with afferent fibers from arterial baroreceptors.

In summary, our results suggest that ET-1 may augment synaptic transmission and may thus facilitate the arterial baroreflex. In agreement with our results, Itoh and Buuse (12) showed that the intracisternal ET-1 administration facilitates arterial baroreflex control of the heart rate. On the other hand, a preliminary report has also shown that microinjection of BQ-123 into the NTS increases the arterial baroreflex sensitivity, thus suggesting that the activation of ETA receptors inhibits the arterial baroreflex (23). Kuwaki et al. (19) have shown that intracisternal ET-1 administration inhibits the arterial baroreflex. In that study, however, ET-1 caused marked hypotension and bradycardia. It is thus difficult to compare the reflex response because of the marked baseline differences in both the arterial pressure and heart rate before and after ET-1. In addition, intracisternal ET-1 also affects other important ET-1-sensitive areas, such as the ventrolateral medulla, more than the NTS (19). Furthermore, we could not determine from our study whether the neurons whose activity was augmented by Glu received inputs only from the arterial baroreceptors. It is possible that they received inputs from chemoreceptors and/or other visceral receptors (29). Further studies are needed to clarify the role of ET-1 on the neurons of the NTS that synapse with afferent fibers from arterial baroreceptors.

In summary, our results suggest that ET-1 increases the neuronal activity of the mNTS via ETA receptors on the postsynaptic membrane while endogenous ET helps control the basal spontaneous neuronal activity of the mNTS. In addition, ET-1 also facilitates synaptic (glutamatergic) transmission in the NTS. Together with the results of other studies in vivo, our results therefore suggest that ET-1 within the NTS may play an important role in the autonomic control of the cardiovascular system.

Perspectives

Interestingly, a recent report (17) has shown that blood pressure is elevated in mice deficient in ET-1, thus suggesting that endogenous ET-1 may have a

![Fig. 5. Effects of ET receptor antagonists on Glu-induced increases in neuronal activity of mNTS. A and B: raw neurogram is shown at top, and firing rate is shown at bottom. BQ-123 but not BQ-788 attenuated Glu-induced increases in neuronal activity of mNTS. C and D: bar graphs showing magnitude of increases in neuronal activity evoked by iontophoretically applied Glu before and during perfusion with BQ-123 (10^{-4} M) (C) or BQ-788 (10^{-4} M) (D). *P < 0.05 vs. values before perfusion with BQ-123 or BQ-788.](image-url)
vaso depressor action. Because ET-1 is a potent vasoconstrictor, this observation was unexpected. They excluded the possibility of an upregulation of vascular receptors for ET and hyper sensitivity to ET-1 because the pressor response to intravenous injection of ET-1 was comparable in heterozygous and wild-type mice. They also excluded the contribution of nitric oxide, the production of which is known to be stimulated by ET-1. Thus a possible site of the depressor action of ET-1 may be the central nervous system. Furthermore, it has also been shown that the ET-1 level in the cerebrospinal fluid is greater than that in the plasma (9, 24). These results suggest that central ET-1 may contribute to the depressor action. However, the cause of hypertension in ET-1-deficient mice will be hard to determine because ET-1 has so many different sites of action in the peripheral and central nervous systems.

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