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TOMOYUKI KUWAKI

桑木 共之

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1. ABSTRACT

In order to characterize effects of endothelin (ET) on the central neural control of the circulation and respiration and to identify neural sites within the central nervous system (CNS) that serve to mediate these effects, ET-1 or ET-3 was administered either systematically into the cisterna magna or topically into certain critical sites of the medulla oblongata in urethane-anesthetized, immobilized male rats under artificial respiration. Furthermore, ET-like immunoreactivity was examined within the medullary sites that were sensitive to ET. Major findings are summarized as follows.

1. An intracisternal injection of 0.1 pmol of ET-1 or 10 pmol of ET-3 in vagotomized rats resulted in immediate increases in arterial pressure (AP), renal sympathetic nerve activity (RSNA, i.e., vasomotor activity), heart rate (HR), phrenic nerve activity (PNA) and the number of bursts of PNA per minute (burst rate, i.e., intrinsic rhythm of the respiratory rhythm generator) that lasted for 5-45 min. At higher doses (≥1 pmol with ET-1 or ≥100 pmol with ET-3) the initial increases (phase I) were followed by decreases below the pre-injection level (phase II) that persisted for 20 to 80 min. Thus, ET affected tonic control of the circulation and respiration.
2. During the latter part of phase I and throughout phase II, ET uniformly suppressed the arterial baroreceptor reflex, peripheral and central chemoreceptor reflexes, and somato-sympathetic reflex. Thus, ET affected reflex control of the circulation and respiration.
3. The change in AP induced by intracisternally administered ET was completely abolished by pretreatment with the ganglion blocking agent, but not with the vasopressin V_1 antagonist, angiotensin II antagonist, or β-blocking agents. Thus, ET-induced cardiovascular changes were subserved predominantly by

sympathetic vasomotor fibers.

4. Subsequently, CNS sites that mediated the ET-induced cardiorespiratory changes were surveyed within the medulla oblongata. Three sites responded to topical application of ET. They were the ventral surface of the medulla oblongata (VSM), nucleus tractus solitarius (NTS) and area postrema. Of these, the VSM was by far the most sensitive to ET.
5. Within the VSM there were two ET-sensitive regions, termed the rostral and caudal ET-sensitive areas, which generated cardiorespiratory changes on topical application of ET. The rostral area coincided with the glycine- and glutamate-sensitive areas and constituted the ventral surface of the rostral ventrolateral medulla (RVLM) where reticulospinal vasomotor neurons were located. Topical application of ET to the rostral ET-sensitive area caused the pattern of responses virtually identical to that following an intracisternal administration of ET. The caudal ET-sensitive area partly overlapped with the nicotine-sensitive area. There, ET caused respiratory changes characterized typically by a marked decrease in PNA with an increase in burst rate.
6. The threshold dose of ET-1 applied to the rostral area to elicit cardiorespiratory changes was as low as 1 fmol. This extremely low threshold dose may mean that this subregion and subjacent RVLM serve as the sensor for ET in the cerebrospinal fluid (CSF). Preliminary results demonstrate responsiveness of vasomotor neurons in the RVLM to topically applied ET to the VSM and intracisternally administered ET.
7. Some RVLM neurons were positively immunostained with anti-ET-antibody along with neurons in the NTS, dorsal motor nucleus of vagus nerve (DMN) and raphe nuclei. Failure to observe ET-like immunoreactivity within nerve fibers or glial cells suggests the role of ET as a neuropeptide in some of these

CNS sites.

These findings are consistent with the following hypothesis. ET circulating in the CSF is monitored by neurons in the rostral ET-sensitive area of the VSM and/or vasomotor neurons in the RVLM and affects tonic and reflex control of the circulation and respiration. Furthermore, ET may serve as a neurotransmitter or neuromodulator in the RVLM and other neural sites that participate critically in central cardiorespiratory control.

2. ABBREVIATIONS

ACSF; artificial cerebrospinal fluid

AP; arterial pressure

Basilar a., BA; basilar artery

CNS; central nervous system

CSF; cerebrospinal fluid

CVLM; caudal ventrolateral medulla

DMN; dorsal motor nucleus of vagus nerve

DRG; dorsal respiratory group

ECG; electrocardiogram

ET; endothelin

HR; heart rate

ICA; inferior cerebellar artery

ION; inferior olivary nucleus

IX; glossopharyngeal nerve

LRN; lateral reticular nucleus

MAP; mean arterial pressure

mRNA; messenger ribonucleic acid

NTS; nucleus tractus solitarius

NV; spinal nucleus of trigeminal nerve

PBS; phosphate buffered saline

PNA; phrenic nerve activity

Pyr; pyramidal tract

RSNA; renal sympathetic nerve activity

RSND; renal sympathetic nerve discharge

RVLM; rostral ventrolateral medulla

V; trigeminal nerve

VLM; ventrolateral medulla

VRG; ventral respiratory group

VSM; ventral surface of the medulla

X; vagus nerve

XII; hypoglossal nerve

3. INTRODUCTION

Endothelin

Endothelin (ET) is a family of biologically active peptides originally isolated from the vascular endothelium of the pig in 1988 and, at present, the only endothelium-derived constricting factor (EDCF) whose chemical structure is determined [Yanagisawa, *et al.*, 1988]. ET family consists of three isoforms, i.e., ET-1, ET-2, ET-3 [Inoue, *et al.*, 1989], and a snake venom, sarafotoxin S6b [Takasaki, *et al.*, 1988]. They all consist of 21 amino acid residues, and are characterized by two disulfide bonds. When ET-1 is intravenously given to rats by a bolus injection, it causes powerful and sustained vasoconstriction even at a dose as small as 1 nmol/kg [Yanagisawa, *et al.*, 1988]. Besides its effect on the vascular smooth muscle cells, ET acts on a variety of tissues such as those of the gastrointestinal tract, kidney, lung, and central and peripheral nervous systems [Yanagisawa and Masaki, 1989]. At least two members of the family, ET-1 and ET-3, exist in the central nervous system (CNS) [Matsumoto, *et al.*, 1989].

Recently, two subtypes of receptors for ET have been identified and termed ET_A and ET_B [Vane, 1990]. They are both rhodopsin type, G-protein coupled molecules with 7 transmembrane domains. The ET_A receptor has high specificity to ET-1 [Arai, *et al.*, 1990], whereas ET_B receptor is non-selective and accepts the three subtypes of ET almost equally [Sakurai, *et al.*, 1990]. Endothelins elicit vasoconstriction mediated by ET_A receptors of the smooth muscle cell and vasodilation mediated by ET_B receptors on the endothelial cell [Sakurai, *et al.*, 1992]. Both receptors are widely distributed over many tissues including the brain [Arai, *et al.*, 1990; Sakurai, *et al.*, 1990].

Endothelin and endothelin receptors in the brain

The messenger RNA of all the three ET isoforms are demonstrated in neurons of the human CNS [Giaid, *et al.*, 1991; Giaid, *et al.*, 1989; Lee, *et al.*, 1990]. Since ET does not penetrate the blood-brain barrier [Koseki, *et al.*, 1989], most, if not all, ET in the CNS seems to be produced there. In fact, activity of the ET-converting enzyme, which catalyzes cleavage of inactive precursor, big-ET, and synthesizes mature ET, is detected in the brain [Hashim and Tadepalli, 1991]. Concentration of ET-1 and ET-3 in the brain tissue (90-160 fmol / g wet tissue and 15-60 fmol / g wet tissue, respectively) is lower than that of atrial natriuretic peptide (1-10 pmol / g) or substance P (20-200 pmol / g) but much higher than that of angiotensin II (a few fmols / g) [Phillips, 1987]. ET-1 and ET-3 are also present in the cerebrospinal fluid (CSF) at the concentration of 0.1 -11 pM and 0.1 - 33 pM, respectively [Ando, *et al.*, 1991; Fujimori, *et al.*, 1990; Kraus, *et al.*, 1991; Shirakami, *et al.*, 1990; Suzuki, *et al.*, 1990; Suzuki, *et al.*, 1990; Togashi, *et al.*, 1990; Yamaji, *et al.*, 1990]. Thus, the concentrations of ET in the CSF is either comparable or even higher than that in plasma (0.4 -1.6 pM for ET-1 and about 0.2 pM for ET-3) [Fujimori, *et al.*, 1990; Kaufman, *et al.*, 1991; Schiffrin and Thibault, 1991; Yamaji, *et al.*, 1990]. ET is distributed in various CNS sites including the pituitary gland, supraoptic and paraventricular nuclei of the hypothalamus, dorsal motor nucleus of the vagus, intermediolateral cell column in the spinal cord, cerebral cortex, striatum and hippocampus [Giaid, *et al.*, 1991; Giaid, *et al.*, 1989; Lee, *et al.*, 1990; Yoshizawa, *et al.*, 1990]. Many of these structures are involved in the cardiorespiratory control [Loewy, 1991]. ET is also present in glial cells of the CNS, although its appearance seems to be limited to certain occasions such as the early developmental stage or during active gliosis that occurs as part of the defence mechanism [Cintra, *et al.*, 1989; MacCumber, *et al.*,

1990].

The messenger RNA of ET_A and ET_B receptors is both expressed in the CNS [Arai, *et al.*, 1990; Sakurai, *et al.*, 1990] and binding sites for [^{125}I]ET-1 and [^{125}I]ET-3 are widely distributed along the neuraxis [Fuxe, *et al.*, 1989; Jones, *et al.*, 1989; Koseki, *et al.*, 1989; Koseki, *et al.*, 1989]. For example, dense binding sites have been found in the circumventricular organs, e.g., the anteroventral 3rd ventricular region (AV3V), which lack the blood-brain barrier and, thereby, have an access to ET in the circulating blood. Supraoptic and paraventricular nuclei also have binding sites for ET. Moreover, cultured anterior pituitary cells express ET-binding sites [Stojilkovic, *et al.*, 1990]. These CNS sites constitute the neuroendocrine system. In the lower brain stem and spinal cord, ET-binding sites are distributed in the area postrema, inferior olivary nucleus, spinal nucleus of the trigeminal nerve, nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMN) [Koseki, *et al.*, 1989]. Most of these CNS sites participate in the central cardiorespiratory control. Especially interesting in this connection is that ET receptors are confirmed in the rat's ventrolateral medulla (VLM) [Gulati and Rebello, 1991], a presumed cardiovascular center at which tonic discharges of sympathetic vasomotor fibers are generated [Guyenet, 1990] (see below). These findings suggest participation of ET in the brain and CSF in the central cardiorespiratory control.

Central neural control of the circulation

There are a number of neural/neurohumoral mechanisms by which the CNS controls the heart and blood vessels. Among them major mechanisms of physiological importance are those mediated by the sympathetic and parasympathetic nerves, the sympatho-adrenal system and the neuroendocrine

system involving secretion of vasopressin [Kumada, *et al.*, 1990]. Furthermore, some regulatory mechanisms are activated secondarily. For example, renin secretion from the juxtaglomerular apparatus is promoted by excitation of the renal sympathetic nerve. Of these multitudes of regulatory mechanisms, the sympathetic nervous system plays the most crucial role in the acute control of the circulation [Kumada, *et al.*, 1990].

One of the fundamental features of the sympathetic nervous system in the control of the circulation is presence of a neuronal mechanism generating basal or tonic discharges on efferent cardiac sympathetic and sympathetic vasomotor fibers [Calaresu and Yardley, 1988] which is conventionally called the "sympathetic cardiovascular center" (or simply "vasomotor center") [Alexander, 1946]. Although there have long been arguments on its localization and neuronal mechanism, it is now generally accepted that the neuronal substrate of the vasomotor center is located within the medulla oblongata [Guyenet, 1990]. More specifically, the rostral ventrolateral medulla (RVLM), a circumscribed area located in the ventrolateral quadrant of the rostral medulla, is the site where tonic sympathetic discharges are generated or, alternatively, constitutes the pivotal site of the neural circuitry generating such discharges. Some of the previous studies that led to this conclusion are as follows.

In 1974, Guertzenstein and Silver [Guertzenstein and Silver, 1974] showed that bilateral application of neuroinhibitory substance, glycine, to a restricted area in the ventral surface of the rostral medulla resulted in a marked decrease in blood pressure to a level equivalent to that found after complete transection of the cervical spinal cord. Because glycine does not affect the axon of passage [Curtis, *et al.*, 1968], they concluded that there must be neural cell bodies within the RVLM, close to the glycine-sensitive area, tonically activating sympathetic vasomotor neurons and hence maintaining the resting blood pressure.

It was subsequently found that tonically active neurons in the RVLM have axonal projections to the sympathetic preganglionic neuron located in the intermediolateral cell column of the thoracic and lumber cords and tonically provide the sympathetic vasomotor outflow to the cardiovascular system (for review, see Ciriello, Caverson, and Polosa [Ciriello, *et al.*, 1989], and Loewy and Spyer [Loewy and Spyer, 1990]). Moreover, using the brain slice of the rat's medulla, some RVLM neurons produced the 'pace maker potential' and generated spontaneous discharges in the complete absence of the excitatory synaptic input [Sun, *et al.*, 1988].

Another feature of the RVLM is that it is a major sites of convergence of inputs arising from peripheral receptors (such as baroreceptors) as well as suprabulbar CNS sites such as the hypothalamus [Kumada, *et al.*, 1990; Kuwaki, *et al.*, 1985; Terui, *et al.*, 1987; Terui, *et al.*, 1986; Terui, *et al.*, 1988]. Namely, the RVLM receives the neuronal input from the NTS, caudal ventrolateral medulla (CVLM), parabrachial nucleus, periaqueductal gray, and hypothalamus all of which participate in the central control of the circulation [Dampney, 1990]. For example, the NTS is the site of termination of primary afferents arising from arterial baro- and chemoreceptors as well as cardiopulmonary receptors [Ciriello, 1983; Kumada and Nakajima, 1972; Miura and Reis, 1969]. Neurons in the CVLM are activated by stimulation of baroreceptors and supply inhibitory inputs to the RVLM [Terui, *et al.*, 1990] and cause sympathoinhibition on activation of arterial baroreceptors. Hypothalamus is a suprabulbar integrative center for neurohumoral control of autonomic functions. Command signals from the hypothalamus mediate the cardiovascular component of complex physiological responses such as those associated with control of the fluid volume and affective behaviors [Spyer, 1989].

Another important function of the RVLM is its sensitivity to various endogenous and exogenous substances. For example, intraparenchimal injection

into the RVLM or iontophoretic application to vasomotor neurons there of catecholamines [Sun and Guyenet, 1986], acetylcholine [Sun and Guyenet, 1986; Sundaram, *et al.*, 1988], angiotensin II [Allen, *et al.*, 1988; Muratani, *et al.*, 1991], enkephalin [Punnen, *et al.*, 1984], serotonin [Lovik, 1989] causes cardiovascular changes mediated by sympathetic vasomotor fibers. The precise mechanism of chemosensitivity of the RVLM is undetermined, although the following two hypotheses are dominant. First, chemosensitivity resides in a neuronal mechanism residing in the VSM which makes the ventral border of the RVLM and is also called the central chemosensitive area [Bruce and Cherniack, 1987]. In fact the VSM does respond to a variety of chemical substances and affects the circulation and respiration (see below). The afferent signal arising at the VSM is transmitted to the vasomotor neurons in the subjacent RVLM. Second, vasomotor neurons in the RVLM themselves are chemosensitive to certain substances. In fact dendrites of these neurons extend to the immediate vicinity of the VSM and possibly respond to chemical stimulation applied to the VSM [Sun, *et al.*, 1991]. Moreover, since RVLM neurons are innervated by nerve terminals containing a variety of substances including L-glutamic acid, γ-aminobutyric acid, noradrenaline and many more, they should possess receptors to these substances [Ciriello, *et al.*, 1986].

As briefly explained above, the neural 'circuitry map' subserving central cardiovascular control has been continuously elaborated though not completed yet. There remains many crucial questions to be solved, especially those concerning the biochemical aspect of the cardiovascular control. Increasing number of endogenous bioactive substances have been reported and suspected to serve as neurotransmitters or neuromodulators. To name but a few, 5-hydroxytryptamine, adrenaline, noradrenaline, acetylcholine, γ-aminobutyric acid, glutamic acid and peptides including substance P, neuropeptide Y, angiotensin II, and enkephalin. Many of these substances have been reported to be contained in the RVLM and proposed to

play some roles in the cardiovascular control [Ciriello, *et al.*, 1986], although our understanding on this subject is inadequate.

Central neural control of the respiration

In 1930's Heymans [Heymans and Neil, 1958] found peripheral chemoreceptors located at the carotid bifurcation and aortic arch. They responded to changes in arterial PO_2, and, to a lesser degree, arterial PCO_2 and pH, and initiated reflex respiratory responses. Existence of the central chemoreceptor was suspected soon after, since, even after complete elimination of the afferent input arising from peripheral chemoreceptors, hypercapnia caused vigorous hyperventilation. Subsequently, Leusen [Leusen, 1950; Leusen, 1954; Leusen, 1954] superfused the brain ventricle of the dog with modified CSF of various concentrations of PCO_2 and pH and elicited respiratory responses. In search for the precise location of the central chemoreceptor, Loesche, Mitchell, and Schläfke [Schlaefke, 1981; Trouth, *et al.*, 1973] eventually identified three subregions of the ventral surface of the medulla oblongata and named them L, M, and S areas [Feldberg, 1976]. They proposed that M and L areas (also called rostral and caudal areas, respectively) were the site of chemoreception, whereas the S area (also called intermediate area) received the input from the other two areas. Thus, cooling or local application of anesthetics to the S area resulted in a profound decrease in the amplitude of phrenic nerve activity with little change in the respiratory rate [Millhorn, 1986]. Therefore, mechanisms to determine the magnitude of respiration and respiratory rhythm are thought to be separate and can operate independently.

Respiratory rhythm, as represented by the burst rate of phrenic nerve activity, is generated by the neuronal mechanism composed of two sets of

medullary neurons designated as the dorsal and ventral respiratory groups (DRG and VRG, respectively). The two groups of neurons are distinguished with respect to the temporal relationship of neuronal activity and that of the phrenic nerve [Ezure, 1990; Kalia, 1981].

Although there still remain a number of basic questions in the field of central neural control of the respiration, I am especially interested in the mechanism of central chemoreception by the VSM. The VSM and the subjacent RVLM are the site at which chemical environment of the CNS is monitored. In fact, application of a wide variety of substances including L-glutamate, glycine, nicotine, acetylcholine, angiotensin II and vasopressin to the VSM resulted in cardiorespiratory responses [Bruce and Cherniack, 1987]. Moreover, as mentioned above, the RVLM is the pivotal site of generation of tonic sympathetic vasomotor discharges and a site of cardiorespiratory coordination [Millhorn and Eldridge, 1986].

4. PURPOSES

As explained above, growing evidence demonstrates that ET acts on the CNS and affect certain functions including the circulation and respiration. It may serve as a neurotransmitter or neuromodulator in some neuronal networks. It is also possible that ET contained in the CSF or extracellular fluid in the CNS is monitored and affects neuronal activity. To have these pieces of background information in mind, I sought (i) to characterize the cardiorespiratory changes induced by centrally and topically administered ET and (ii) to identify CNS sites that mediate these changes.

Specifically, I sought to answer the following questions.

(1) What is the effect of intracisternal administration of ET on the circulation and respiration. Does the agent affect tonic CNS control of cardiorespiratory functions or does it modulate reflex control as well?

(2) What is the efferent mechanism of cardiovascular changes induced by centrally administered ET? Are they mediated by the sympathetic nervous system exclusively or are adrenal and vasopressin secretion critically involved?

(3) Which CNS sites mediate the central cardiorespiratory effects of ET? Are these sites located in the medulla oblongata and below along the neuraxis or are suprabulbar structures also critically involved?

(4) What is the role of the VSM and subjacent RVLM in the cardiorespiratory changes induced by centrally or topically applied ET? Within the VSM is there any special subregion(s) which is sensitive to ET? Do vasomotor neurons in the RVLM participate in the ET-induced cardiovascular changes?

(5) Are the CNS sites responsive to ET positively immunostained with anti-ET-antibody so that ET may serve as a neurotransmitter or a neuromodulator?

5. METHODS

5.1 Preparation of Animals

Animals, anesthesia and surgical procedures

Experiments were performed on 11-13 weeks old male Sprague-Dawley rats weighing between 300 and 460 g. They were anesthetized, except in 17 rats noted below, with an intraperitoneal injection of urethane (initially 1 g/kg). Supplementary doses were intravenously given when required as judged from recordings of arterial pressure (AP) and ventilation. For the purpose of comparing the effect of different anesthetics, eight rats were anesthetized by an intraperitoneal injection of α-chloralose (100 mg/kg, initial dose) or sodium pentobarbital (60 mg/kg, initial dose).

After insertion of tracheal, arterial and venous cannulas, the animal was paralyzed with gallamine triethiodide (initially 5 mg/rat i.v., thereafter 6 - 8 mg/h, i.v. with infusion pump; Harvard Apparatus, 975E) and artificially ventilated (Harvard Apparatus, Rodent Respirator C80E) with oxygen-enriched room air. End-tidal P_{CO_2} was continuously monitored (Sanei-NEC, Respina IH26) and maintained between 3 and 4.5% by adjusting ventilation volume and frequency [Kleinman and Radford, 1964]. Airway pressure was continuously monitored from a side tube of the mouthpiece that was connected to a pressure transducer (Nihon Kohden, TP-200T) to check effectiveness of anesthesia and immobilization. Body temperature was monitored by a thermistor probe (Yellowsprings Instrument, 401) inserted into the rectum and maintained between 37 ± 0.5 °C by a thermostatically regulated DC heating pad.

After introduction of paralysis, the animal's head was placed in the stereotaxic

frame (Narishige, ST-7) in spine or prone position according to the experiment.

Precollicular transection of the brain

In nine rats, unanesthetized animal preparations were made by transecting the brainstem at the precollicular level. Under halothane anesthesia (2%), a parietal craniotomy was performed by perforating a hole with a dental drill at a point approximately 1 mm rostral to the lambdoid suture and 1 mm left to the midline. Air emboli were protected by dropping saline during drilling, and with bone wax. The hole was then enlarged laterally and rostrally. Part of an occipital lobe was aspirated so that a spatula was inserted into the midbrain to perform a precollicular transection. After the brain tissue rostral to the brain section was removed by aspiration and the remaining cavity was filled with gelatin sponge (Yamanouchi Pharmaceutical, Spongel), the anesthesia was discontinued. During precollicular transection and following aspiration, bilateral common carotid arteries were clumped to lessen bleeding. After the anesthesia was discontinued, a 2-h intermission was interposed to eliminate the effect of halothane.

Measurement of arterial blood pressure and heart rate

Instantaneous and mean arterial pressures, heart rate (HR), and electrocardiogram were monitored continuously in all experiments. AP was recorded from the abdominal aorta by a polyethylene catheter (0.5 mm i.d.) inserted through the femoral artery and connected via a transducer (Nihon Kohden, TP-200T) to an amplifier (Nihon Kohden, AP-620G). Pulsatile AP was continuously displayed on a polygraph (Nihon Kohden, RM-6000), and mean AP (MAP) was recorded on a second channel by damping (0.2 Hz low pass) the phasic

signal. HR was computed from the AP pulse by a tachometer (Nihon Kohden, AT-601G) and displayed on a third channel.

5.2 Recording of Renal Sympathetic and Phrenic Nerve Activities and Single Unit Activity of Vasomotor Neurons

The left renal sympathetic nerve was approached retroperitoneally and prepared for recording from near the renal artery. To record its efferent discharges, the central cut end of the nerve was placed on bipolar silver hook electrodes connected to an amplifier (Nihon Kohden, AVB-8) and displayed on an oscilloscope (Tektronix, 5113). The lower and upper cut-off (-3 dB) frequencies of the recording system was 100 and 3000 Hz, respectively. The nerve was immersed in warm paraffin oil to avoid drying.

Renal sympathetic nerve discharges were quantitated by one of the following methods. First, discharges were full-wave rectified, integrated over a 10 s-interval (Nihon Kohden, EI-601G) and stored in a tape recorder (TEAC, SR-31DF) together with mean AP (MAP), HR, ECG (lead II), and timing pulses of drug injection or other event signals. At the end of the experiment, the renal nerve was cut proximally to the recording electrode and the level of the instrumentation noise was determined. The noise level thus determined was integrated over 10-s interval for a few minutes and the average value of the integrated noise level was calculated. In some animals, before cutting the renal nerve, contribution of preganglionic nerve activity in the recorded renal nerve discharge was determined with ganglion blockade by intravenous injection of hexamethonium chloride (10 mg/kg). The remaining signal after ganglion blockade was almost same as the noise level, indicating that almost all of the recorded nerve signals were originated from the sympathetic postganglionic nerve fibers. When the recorded nerve signals were

reproduced for processing, the rectified and integrated renal nerve discharges were subtracted by the integrated and averaged noise level. The second method was to convert renal nerve discharges into standard pulses by a window discriminator (Nihon Kohden, EN-601J) at the time of reproducing the recorded signals and determine the discharge frequency by a frequency counter (Nihon Kohden, ET-612J). To obtain standard pulses from nerve discharges, the threshold was set slightly above the noise level. The first method was used in the majority of experiment, especially in all that dealt with the response magnitude. Renal sympathetic nerve discharges quantitated in this manner were called renal sympathetic nerve activity (RSNA). RSNA was used as a measure of sympathetic vasomotor activity, since it accurately reflects activity of sympathetic vasoconstrictor fibers [Dorward, *et al.*, 1986].

Phrenic nerve was prepared for recording, depending on the protocol, in one of the following two ways. When the ventral surface of the medulla was exposed for drug delivery (see next section) and the animal was placed in spine position in the stereotaxic frame, right phrenic nerve was approached ventrally by separating neck muscles. In all the other experiments, in which the animals were placed in prone position, left phrenic nerve was prepared for recording via dorsolateral approach. After the acromiotrapezius muscle was divided longitudinally, the phrenic nerve was isolated from the ventral division of the fifth cervical plexus. The nerve was cut distally and placed on a bipolar silver hook electrode. The nerve and electrode were immersed in warm paraffin oil. The recorded multifiber phrenic nerve discharges were processed as with renal nerve discharges to obtain phrenic nerve activity (PNA). The vagus nerve was cut bilaterally in the neck to avoid Hering-Breuer reflex and so that PNA represented inspiratory activity of the central respiratory rhythm generator. The aortic and carotid sinus nerves remained intact. It has been known that multifiber phrenic nerve discharges

increase abruptly at the onset of the inspiratory phase from a background of little or no activity, keep increasing and then rapidly diminish at the termination of this phase, although a much smaller portion of them sometimes continue into the postinspiratory phase [Berger and Hornbein, 1989; Richter, 1982]. In the present experiments in vagotomized rats, PNA was thus used to assess activity of the central respiratory rhythm generator. Burst rate of PNA, corresponding to the respiratory frequency in vagus-intact rats, was calculated by passing the rectified multifiber discharges through a low pass filter with the cut-off frequency of 2 Hz. The signal thus processed was then fed into a pulse counter whose threshold was set just above the noise level.

For recording of single unit activity of vasomotor neurons in the RVLM, the animals were placed prone in a stereotaxic frame and the spinal cord was secured using a vertebral clump placed at Th2 - Th3. A small hole, about 3 mm in diameter, was then drilled at the suture between interparietal and occipital bones and the dura overlying the cerebellum was removed. A glass microelectrode (o.d. ~ 1.4 mm), filled with 0.5 M sodium acetate containing 2% pontamine sky blue, was inserted through the cerebellum and advanced to the RVLM using a pulse motor drive (Narishige, MO-81) to record extracellular single unit activity. The impedance of the microelectrode measured at 5 Hz was 4 - 10 MΩ. A reference electrode made of the brass plate was attached to the neck muscle. Neuronal signals thus recorded were amplified and filtered (Nihon Kohden, MEZ 8201, band-pass frequencies: 50 - 3000 Hz), monitored on an oscilloscope (Nihon Kohden, VC-11) and stored on a tape recorder (TEAC, XR-7000L). The signal, digitized by a window discriminator, was counted during a 1-s period and recorded as integrated neuronal activity.

Vasomotor neurons in the RVLM were searched as follows. The electrode was inserted at the point 1 mm caudal to the external occipital protuberance and 2

mm lateral to the midline. The electrode was first advanced to the position around the nucleus ambiguus so that the discharges of neurons in the ventral respiratory group were recorded. The RVLM and vasomotor neurons there were located in the area between this neuronal group and the ventral surface of the medulla [Guyenet, 1990]. Precise location of the recorded neuron was marked at the end of the experiment with a deposit of pontamine sky blue dye made by passing a train of cathodal pulse current of 20 μA intensity and 500 ms duration at 1 Hz for 10 min.

Criteria for identification of vasomotor neurons in the RVLM are as follows. (1) They are located in the RVLM. (2) They periodically discharge synchronous with the cardiac rhythm. (3) Their activity are inhibited by a rise in arterial pressure caused by intravenous phenylephrine. (4) Their spontaneous discharges are masked by antidromic activation of the descending spinal axons (collision test [Terui, *et al.*, 1986]). For antidromic activation, a monopolar stainless steel electrode (Transidyne General Corp.) insulated with enamel lacquer except 0.2 mm at the tip was inserted into the spinal cord at C6 - C7 with its tip 0.7 - 0.9 mm below the dorsolateral sulcus. Placement of the tip at the excitatory spinal sympathetic tract in the dorsolateral funiculus [Illert and Gabriel, 1972] was confirmed by applying a train of rectangular cathodal currents of 10 μA intensity and 0.5 ms duration at 100 Hz for 10 s so that the resulting pressor response was over 30 mmHg. It was further confirmed by marking the tip position with a lesion made by passing a DC current of 2 mA intensity for 10 s at the end of the experiment and subsequent histological examination.

5.3 Administration of Endothelin to the Brain

ET-1 or ET-3 (Peptide Institute, Osaka) was first dissolved into artificial cerebrospinal fluid (ACSF, pH=7.4) [Lindvall, *et al.*, 1978] at a concentration of

10^{-4} M and aliquotes of it were stored at -20 °C before use. The ACSF contained (in mM): 123 NaCl; 3.0 KCl; 0.89 $MgCl_2$; 0.86 $CaCl_2$; 25 $NaHCO_3$; 0.5 NaH_2PO_4; 0.25 Na_2HPO_4.

Intracisternal administration of endothelin

Rats were placed prone in a stereotaxic frame with the bite bar set at -12 mm below interaural line. Under a dissection microscope (Nagashima, MD-2), the needle of a microsyringe (Terumo, MS-N10) carried in a stereotaxic micromanipulator (Narishige, BE-8) was advanced through the exposed atlanto-occipital membrane and the tip was placed within the cisterna magna. Incision was secured with cyanoacrylate adhesive (Toh-a, aronalpha) to avoid back flow of the injected solution. Either vehicle (ACSF), ET-1 or ET-3 (Peptide Institute, Osaka) dissolved in 10 µl of ACSF was injected into the cisterna magna. The injected solution contained 0.5% (w/v) of Evans Blue (Wako) to check, after the experiment, the extent of the brain area where the drug solution reached. Doses of ET-1 employed for the intracisternal injection were 0.1, 1, or 10 pmol and that for ET-3 were 10 or 100 pmol. Injections were made by hand in less than 1 min.

Topical application of ET to the nucleus tractus solitarius (NTS) and area postrema

Rats were placed prone in a stereotaxic frame. After exposure of the atlanto-occipital membrane (see above), it was cut along with the center line and retracted laterally. A part of the occipital bone was removed and in some case, the cerebellum was retracted rostrally until a part of the floor of the fourth ventricle could be seen. The spinous processes of the fifth or sixth thoracic vertebra were fixed to the stereotaxic flame by a brass hook to keep animal's longitudinal axis

straight and to avoid it's rotation around the axis.

ET was injected into the NTS, area postrema, or other medullary sites (see results) through micropipettes fabricated from calibrated glass capillary tubing (Hirschmann, Duran Ringcaps 1 - 5 µl) with tips (ca. 25 µm) shaped by a micropipette puller (Narishige, PE-2). The pipettes were carried in a stereotaxic micromanipulator (Narishige, SM-11) and connected by silicon tubing to the pressure source of 0.4 - 1.5 atm. While observing the fluid meniscus in the micropipette through a dissection microscope equipped with an ocular micrometer, the desired volume was injected by adjusting manually the opening time of a solenoid valve (CKD Corp., AG31021), placed in the pneumatic circuit [Lipski, *et al.*, 1988; Palmer, 1985]. Calamus scriptorius was defined as stereotaxic zero point for rostro-caudal and lateral displacement of the pipette. Dorso-ventral coordinates were defined as the distance ventral to the dorsal surface of the medulla at the point of entry of the pipette. The volume of the injected fluid was 0.2 µl and the injection time was 5 - 10 s. The pipette remained at the injection site during the entire recording period. Exposed dorsal surface of the medulla around the pipette was covered with a piece of cotton wool soaked with saline to prevent drying of the brain.

In six rats ET was delivered to the area postrema via blood stream through the left vertebral artery which was exposed between the foramen of the transverse process of the atlas and that of the axis and was cannulated with a polyethylene catheter (o.d. = 0.5 mm; Natsume, SP8). ET dissolved in saline was given in a volume of 10 µl from a microsyringe connected to the catheter and was flushed by 15 µl of physiological saline. After completion of the experiment, 25 µl of saline containing Evans Blue (1%) was injected to check whether ET was properly delivered.

Topical application of ET to the ventral surface of the medulla (VSM)

Three methods were employed for topical application of ET to the VSM. First, the animals were placed prone in a stereotaxic frame. A glass pipette was penetrated at the exposed dorsal medullary surface (see above), advanced ventrally, passed through the brainstem, and placed in the subarachnoid space below the area of VSM. Pipette shanks were long enough to produce minimal distortion of the medulla. The pipettes were connected by silicon tubing to a microsyringe (Terumo, MS-N10). Injections of the volume of 1 - 5 µl were made manually in less than 30 s.

Second, the VSM was exposed through a ventral approach. The animal's head was fixed to a stereotaxic frame in a supine position with the bite bar set at 10 mm above interaural line to make the VSM horizontal. After the tracheal cannula was inserted, the trachea and esophagus were cut high in the neck between the two ligatures. The longus capitis muscle was removed on both sides at its attachment to the occipital bone. By carefully removing the basilar part of the occipital bone under the dissection microscope, the VSM was exposed by incising the dura and the arachnoid. The exposed VSM was chemically stimulated by topical application of ET soaked into a piece of filter paper. The piece, about 1 x 1 or 2 x 2 mm in size, applied unilaterally to the area rostral to the root of the XIIth cranial nerve.

In some experiment where single-unit activity of the vasomotor neuron was recorded, ET-1 (0.1 or 1 pmol) dissolved in 5 or 10 µl of ACSF was administered to the VSM through a polyethylene catheter (o.d. = 0.5 mm; Natsume, SP8) passed into the subarachnoid space via an incision between the atlas and the ventral part of the occipital bone. It was advanced rostrally for 2 - 3 mm so that the tip lay just caudal to the rostral ET-sensitive area (see results) in the VSM.

Topical application of ET to the subdivisions of the VSM

The VSM was exposed by the ventral approach as explained above. ET-1 and ET-3 were both dissolved in the ACSF which contained 0.5% of Evans Blue to check the extent of the brain surface where the drug solution reached by diffusion. The solution was applied topically to the VSM through a microsyringe (Hamilton Co., Nevada, U.S.A., 7000.5 N) whose flat needle tip (i.d. ~100 μm) was pressed gently on the VSM. In a fixed volume of 0.2 μl, ET-1 was delivered at doses of 0.1, 1, 10, and 100 fmol and 1 pmol, whereas ET-3 was given at 100 fmol, and 1 and 10 pmol. In a similar way, 50 nmol of L-glutamate (in 0.1 μl), 270 nmol of glycine (in 0.2 μl) or 250 nmol of nicotine (in 0.2 μl), all dissolved in artificial cerebrospinal fluid, were applied to the VSM unilaterally. In each instance the drug solution was delivered in less than 5 s. The needle was placed in that position for another 2 to 3 min and was then removed. No effort was made to wash out the drug thus applied. The surface area reached by the injected solution, as judged by simultaneously injected Evans Blue, was within 1 mm from the injected site. On histological examination, the dye did not visibly penetrate deep into the brain stem from the ventral surface. In each experiment, ET was administered up to three times after the effect of previous administration had completely disappeared.

The point of drug applications was positioned stereotaxically. Rostro-caudal and lateral coordinates of each point were determined with reference to the caudal end of the basilar artery which could be positioned more accurately in rats than the median rootlet of the XIIth cranial nerve conventionally used in cats [Trouth, *et al.*, 1973]. Coordinates of the area explored for cardiorespiratory responses to application of ETs and other drugs were between -1.0 and +5.5 mm rostro-caudally and between 0 and 3 mm laterally.

The general scheme of experiments consisted of three parts. First, different

patterns of cardiorespiratory changes were systematically explored in 30 rats by applying 1 pmol of ET-1 or 10 pmol of ET-3 to 59 points in the VSM, while recording MAP, RSNA, PNA, burst rate and HR. When multiple points were tested in the same animal for cardiorespiratory responses, the site of ET application was separated by more than 2 mm among each other. Second, to correlate distribution of ET-sensitive points with the known subdivisions of the VSM with respect to their chemical sensitivity, the cardiorespiratory effect of topical application of L-glutamate (11 rats), glycine (5 rats) or nicotine (6 rats) was examined. Thirdly, after confirming that the glutamate-sensitive area of the VSM was most prominent in eliciting ET-induced cardiorespiratory changes, the dose-response relationship was determined by delivering ET-1 to that area at 5 doses (22 rats) and compared it with that of ET-3 given at 3 doses (8 rats). In preliminary experiments, the magnitude of cardiorespiratory responses tended to be attenuated on repeated application of ET to the same point in the VSM. Therefore, the same dosage was not repeated in a single animal.

Administration of ET to the spinal cord (intrathecal administration)

ET was given to the spinal cord by intrathecal administration in a volume of 10 µl through a polyethylene catheter (o.d. = 0.5 mm; Natsume, SP8) passed into the subarachnoid space via an incision in the atlanto-occipital membrane [Yaksh and Rudy, 1976]. It was advanced caudally for 5 - 7 cm so that the tip lay at Th5 - L1, where preganglionic neurons governing renal sympathetic nerves were located [Strack, *et al.*, 1988].

5.4 Pharmacological Block of Sympathetic Outflow, Angiotensin II, Vasopressin, or β-adrenergic Receptors

To examine the mechanism underlay the change in AP induced by intracisternally administered ET, peripheral sympathetic nervous activity, angiotensin II receptor, or vasopressin receptor was pharmacologically blocked.

Sympathetic ganglion was blocked by continuous intravenous infusion of hexamethonium chloride at a rate of 30 mg/(kg·h) after an initial dose of 30 mg/kg. After AP reach at a stable level near 50 mmHg, ET was intracisternally administrated as described.

Peripheral angiotensin receptor was blocked by i.v. infusion of salarasin (12 μg/(kg·min); Peptide Institute, Osaka). The antagonistic action was confirmed as follows. After the antagonist was given to the animal, angiotensin II was intravenously given as a bolus up to 0.5 μg/kg before and after ET-treatment. The lack of a pressor response was the criterion for the antagonistic effect.

Arginine vasopressin antagonist, (1-(β-mercapto-β,β-cyclopentamethylene-propionic acid),2-(O-methyl)-tyrosine)arginine-vasopressin (Manning compound; Peptide Institute, Osaka) was intravenously (10 μg/kg) administered in separate four rats before intracisternal injection of ET. The antagonistic action was confirmed as above by examining the lack of a pressor response to a bolus injection of arginine vasopressin by up to 0.1 μg/kg.

In another 4 experiments, atenolol (3 mg/kg, n=1), propranolol (3 mg/kg, n=2), or timolol (3 mg/kg, n=1) was injected intravenously before intracisternal injection of ET.

5.5 Elicitation of Arterial Baroreceptor, Central and Peripheral Chemoreceptor, and Somato-sympathetic Reflexes

Arterial baroreceptor reflex

The arterial baroreceptor reflex was elicited by one of the following three method and was compared before and after intracisternal administration of ET-1 or ET-3. (i) The amplitude of the cardiac-related changes of the rectified renal sympathetic discharges was used to assess the reflex activity. In this type of experiment, the post-R wave time histogram of rectified renal sympathetic discharges was constructed by averaging them in 32 - 64 successive sweeps (Nihon Kohden, ATAC 350). The cardiac-related changes in sympathetic discharges are known to represent baroreceptor-initiated sympathoinhibition [Gebber and Barman, 1980]. Since this method is valid as far as levels of AP and RSNA would not change drastically, it was applied to examine the reflex activity during phase I. (ii) During phase II , when AP often diminished below the threshold pressure of the arterial baroreceptor reflex, the reflex activity was assessed by the change in RSNA in response to a bolus i.v. injection of phenylephrine (1-10 μg) (see RESULTS for definition of phases I and II.). (iii) In one case, to exclude possible effect of ET on baroreceptor itself, right aortic nerve, which consisted exclusively of baroreceptor fibers [Numao, *et al.*, 1985; Sapru, *et al.*, 1981], was electrically stimulated and the reflex inhibition of renal sympathetic nerve discharges was examined. In this case, right aortic nerve was dissected in the neck and stimulated at it's distal cut end at 10 V, 100 Hz (pulse duration 0.5 ms) for 1 s. Peri-stimulus time histogram was constructed by counting standardized pulses of renal sympathetic nerve discharges for successive 16 stimulations. The aortic nerve was first stimulated at 10 V, 100 Hz for 10 s and reflex depression was confirmed.

Central and peripheral chemoreceptor reflexes

The VSM and deep structure below the VSM are known as the central chemoreceptive zone by which the change in P_{CO_2} and/or pH is detected [Bruce and Cherniack, 1987]. To stimulate central chemoreceptive zone to elicit central chemoreceptor reflex, hypercapnia was introduced by inhalation with 5% CO_2 - 95% O_2 for 3 min. During the inhalation, the end-tidal P_{CO_2} usually increased by 3 ~ 4 % to reach the final value of 6 ~ 7%. The reflex increase in PNA was compared before and after intracisternal administration of ET. In this series of experiment, some animals were ventilated with 100 % O_2 throughout the experiment instead of the mixture of room air and O_2 so that the reflex increase in PNA could be easily detected. On the other hand, the arterial P_{O_2} is detected by peripheral chemoreceptors of the carotid body located at the carotid bifurcation [Fidone and Gonzalez, 1986]. To induce the peripheral chemoreceptor reflex, the animal was inhaled with 1:1 mixture of the room air and 100% nitrogen for 1 min. During this hypoxic period, the end-tidal P_{O_2} usually decreased to 6 ~ 8 %. The increase in PNA was taken as a measure of the peripheral chemoreceptor reflex.

Somato-sympathetic reflex

The left sural nerve was exposed by removing the femoris biceps muscle and prepared for electrical stimulation near the gastrocnemius muscle [Greene, 1963]. The distal cut end was placed on a pair of silver hook electrode and immersed in a warm paraffin pool which was surrounded by the retracted skins. To activate groups II (or Aβ) and III (or Aδ) fibers jointly, two negative rectangular pulse with the intensity of 5 V and the pulse duration of 0.2 ms were applied at 100 Hz.

To activate groups II, III, and IV (or C) fibers jointly, these stimulus parameters were 30 V, 0.5 ms and 40 Hz. The peri-stimulus time histogram of rectified renal sympathetic discharges (RSND) was obtained by averaging sweeps of RSND during successive 32 stimulations applied every 4 ~ 8 sec.

5.6 Data Analysis and Statistical Methods

While reproducing MAP, HR, RSNA, PNA, and burst rate, they were sampled at the rate of 1 Hz by an analog-to-digital converter (CANOPUS Electronics, ADX-98H). The mean values of these variables over the period of successive 1- or 5-min intervals were then calculated by a computer (NEC PC- 9801RX). The baseline level was defined as the average of each variable over the 10-min interval immediately before administration of ETs.

Statistical analysis of the results was carried out using Student's t-test for paired data before and after administration of ET or for unpaired data between two groups of experiments. To compare the slope of the regression line, the analysis of covariance was used. Differences in data were considered to be significant when $P<0.05$. Results were expressed as mean ± S.E.

5.7 Histological Examinations

General histological procedures

In experiments in which ET was injected into the NTS and area postrema or single unit activity of vasomotor neuron was recorded, the animal was deeply anesthetized and perfused through the heart with saline followed by 4% formaldehyde at the end of the each experiment. The brain was sectioned coronally

at 50 µm thickness on a vibrating microtome (Dohsaka EM, DTK-1000). The sites of dye were identified and related to histological structures [Paxinos and Watson, 1986] before and after staining the section with cresyl violet or neutral red. In experiments in which ET was applied to the subarachnoid space or the VSM, the animal was killed with an intravenous injection of potassium chloride and the brain was excised. Distribution of the injected dye was then visually examined.

Immunohistochemistry

In a separate series of five experiments, distribution of ET-like immunoreactivity in the medulla oblongata was examined. Male Sprague Dawley rats of 8 - 9 weeks old were used. They were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.), heparinized (160 units/kg) and perfused through the left ventricle with 100 ml of saline followed by 250 ml of Bouin's fixative which contained 1 % picric acid, 10 % formaldehyde, and 5% acetic acid. In preliminary experiments, I found that this solution caused most intense staining among the five fixers tested, i.e., 4% paraformaldehyde with or without 0.2% tannic acid, 0.5% glutaraldehyde-2% paraformaldehyde added by Zamboni's and Bouin's fixatives.

The medulla was subsequently removed and placed in the same fixative for 8-16 h followed by 0.1 M phosphate buffer containing 15 % w/v sucrose up to 18 h at 4 °C. Frozen sections of 10-16 µm thick, cut on a cryostat, were mounted on poly-L-lysine (Sigma) coated slides and dried at the room temperature for more than 2 h. After treatment with 0.3 % H_2O_2 in methanol for 30 min at 4 °C, sections were incubated with 1.5 % normal goat serum in 0.01 M phosphate buffered saline (PBS) (pH 7.2) containing 0.3 % Triton X-100 for 20 min at room temperature. Then sections were incubated with monoclonal antibody purified from ET-1-immunized mouse ascites (KY-ET-1-I, a gift from Dr. K. Nakao, Kyoto University [Saito, *et*

al., 1990]) using protein A affinity column (Amersham Japan, Ampure PA) or with anti-ET antiserum raised in rabbits (Peptide Institute) diluted 1/100 - 1/1000 in PBS containing 1.5 % normal goat serum and 0.3 % Triton X-100 for 48 h at 4 °C. Cross-reactivities of KY-ET-1-I, monoclonal antibody to ET, were 100 % to ET-2, 60 % to ET-3, and 100 % to human big ET-1 [Saito, *et al.*, 1990], whereas those of anti-ET antiserum were 100 % to ET-2, 100 % to ET-3, less than 1 % to big ET-1 (porcine, 1 - 39), and less than 1 % to big ET-1 (human, 1 - 38) (manufacturer's manual). Anti-ET antiserum did not cross react with any of the following peptides (1 - 10 nmol/ml); peptide YY, calcitonin gene related peptide (CGRP, human), secretin (human), somatostatin, nor β-endorphin (human). The sections were then immunostained using the avidin-biotin complex (ABC) method as explained in the manufacture's manual (Vectastain Elite Kit, Vector Labs. Inc., Welwyn Garden City, Herts, UK). The peroxidase reaction was developed using the DAB-method or glucose-nickel-DAB method [Shu, *et al.*, 1988]. Sections were subsequently rinsed in tap water, dehydrated and mounted.

6. RESULTS

6.1 Effects of Intracisternal Administration of Endothelin-1 and Endothelin-3 on Central Cardiorespiratory Control

Effects of intracisternal injection of ET-1 and ET-3 on AP, RSNA, HR, PNA and burst rate

In early series of the experiment, the animal was not vagotomized and only cardiovascular parameters, namely AP, RSNA, and HR, were recorded. Later, the animal was vagotomized and respiratory parameters, PNA and burst rate, were also added. Since there was no significant difference in the response of AP, RSNA and HR to intracisternally administered ET between the two sets of experiments (for example, compare Figure 2A and 2B), results from the two groups were pooled and treated as a whole.

An intracisternal injection of 0.1 pmol of ET-1 resulted in immediate and modest increases in AP, RSNA, HR, PNA and burst rate (Figure 1A and 3 and Table 1). The response lasted for 5- 45 min and each variable returned thereafter to the pre-injection level. The duration of the ET-induced pressor response was 21 ± 6 min (n = 8). This period of initial increases was termed phase I. After the increases subsided, PNA and burst rate usually fell below the pre-injection level for the next 5-20 min. At the dose of 1 or 10 pmol, however, the phase I was curtailed by sudden falls in all the variables below the control level (phase II; Figure 1B, 3, 4A, and 4B and Table 1). Especially striking was inhibition of the respiration. Namely, in most experiments, PNA and its burst rate were completely eliminated for a while (Figure 4B), demonstrating disappearance of the centrally generated respiratory rhythm. Phase II was usually terminated in 20 - 80 min and turned into

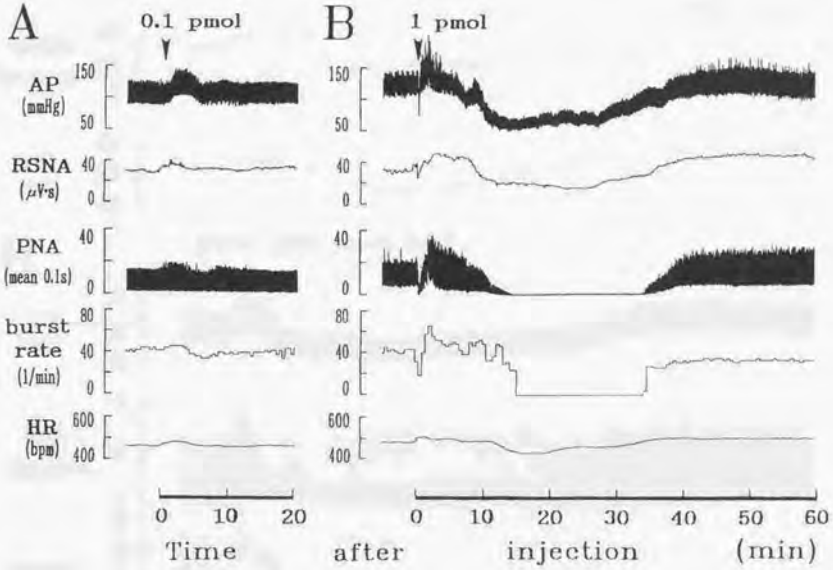


Figure 1 Polygraph records illustrating responses of arterial pressure (AP), renal sympathetic nerve activity (RSNA), phrenic nerve activity (PNA), burst rate of PNA, and heart rate (HR) to intracisternal administration of 0.1 pmol (A) or 1 pmol (B) of ET-1 applied at the moment marked by arrowheads.

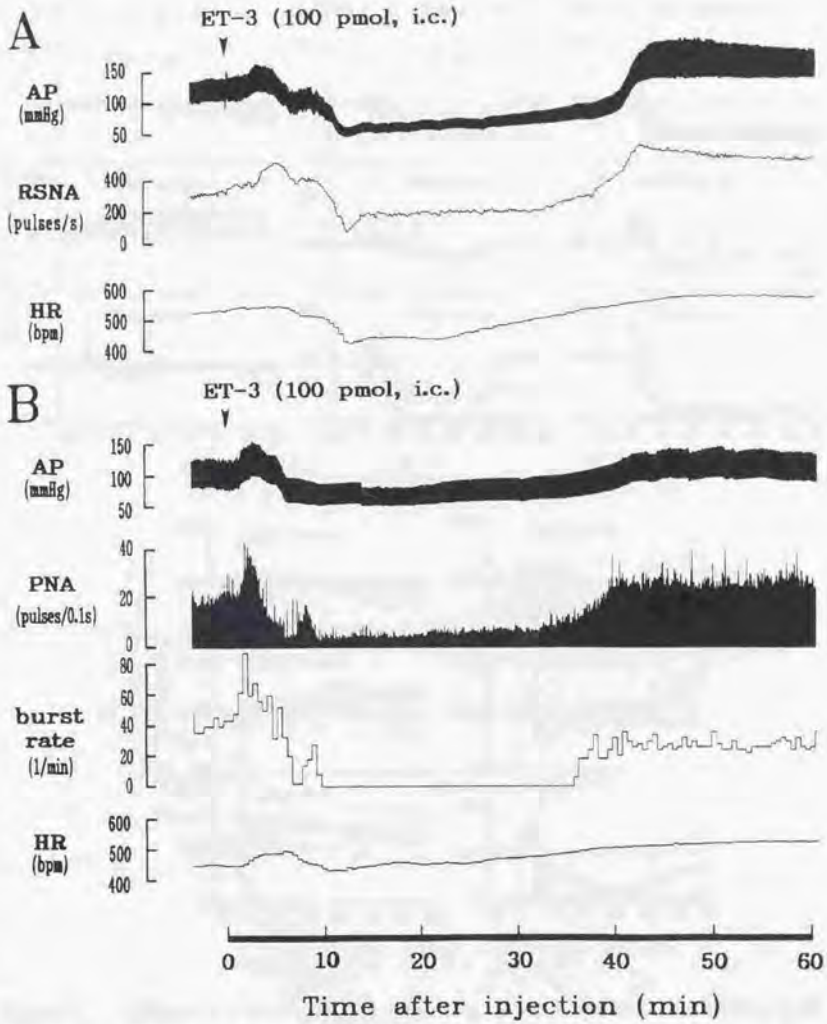


Figure 2 Polygraph records illustrating responses of cardiovascular, sympathetic, and respiratory responses to intracisternal (i.c.) administration of 100 pmol of ET-3 applied in two rats at the moment marked by arrowheads. Arterial pressure (AP) and heart rate (HR) were recorded in both cases, whereas renal sympathetic nerve activity (RSNA) was recorded in A, and phrenic nerve activity (PNA) and burst rate of PNA were recorded in B only.

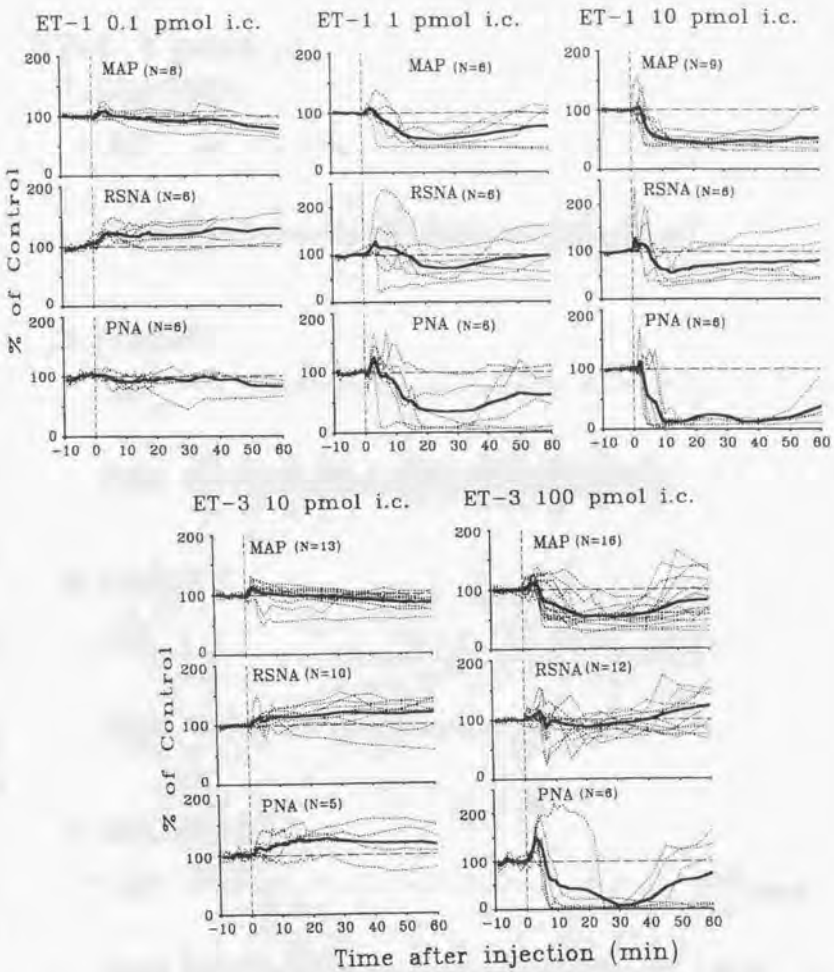


Figure 3 Effects, on MAP, RSNA, and PNA, of i.c. administration of 0.1, 1, or 10 pmol of ET-1 (upper three panels) or 10 or 100 pmol of ET-3 (lower panels). Responses observed in individual animals are represented by dotted lines, whereas the average values at each moment are represented by heavy lines. In this figure, all the variables are expressed as percent of the baseline values determined as the average over 10 min immediately before the drug administration.

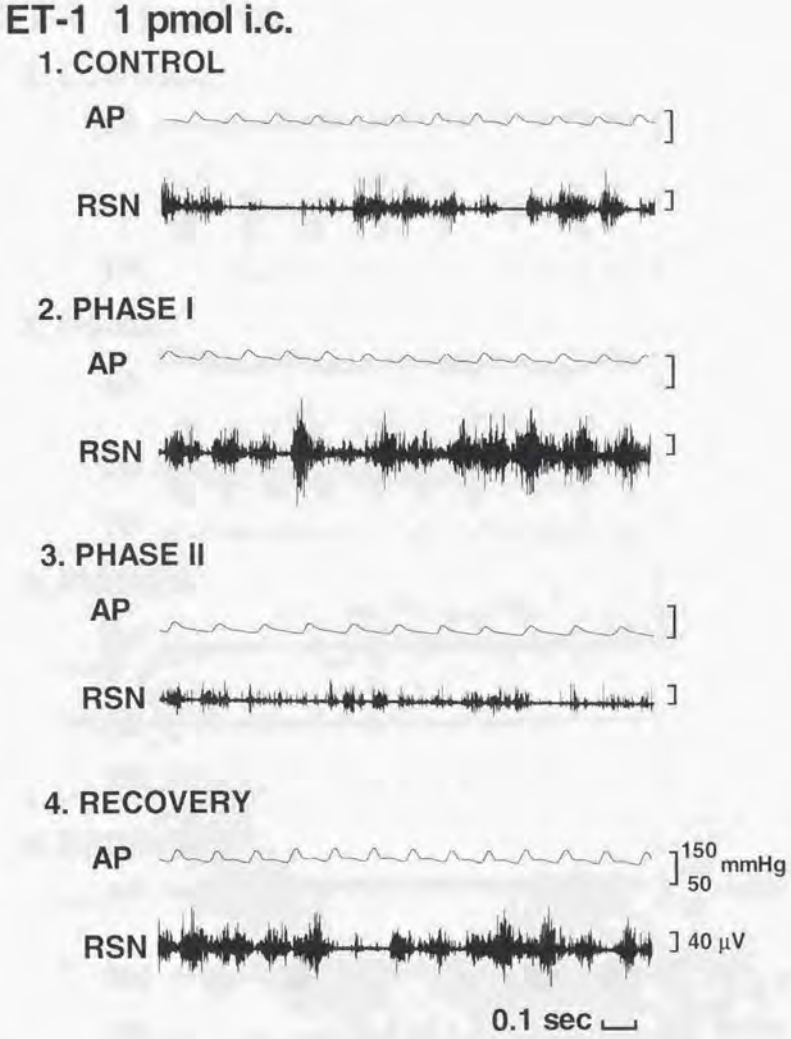


Figure 4A Effects on arterial pressure (AP) and renal sympathetic nerve discharges (RSN) of intracisternal administration of 1 pmol of ET-1 shown in enlarged time scale.

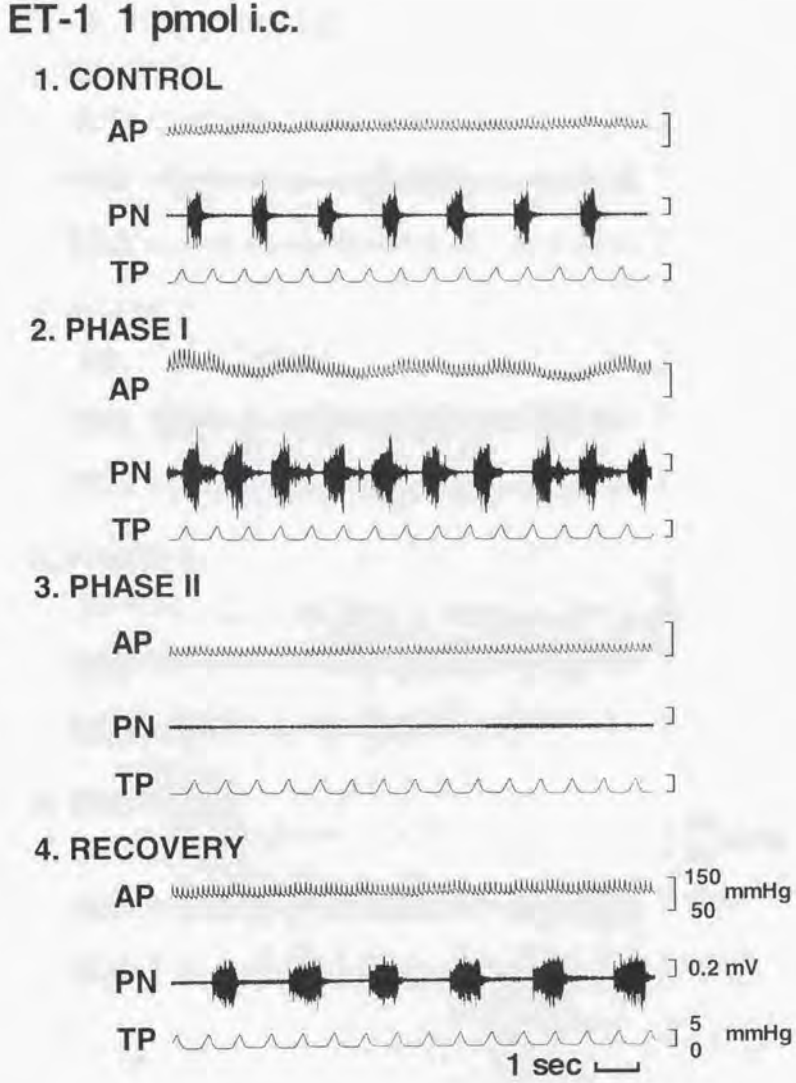


Figure 4B Effects on arterial pressure (AP) and phrenic nerve discharges (PN) of intracisternal administration of 1 pmol of ET-1 shown in enlarged time scale along with tracheal pressure (TP).

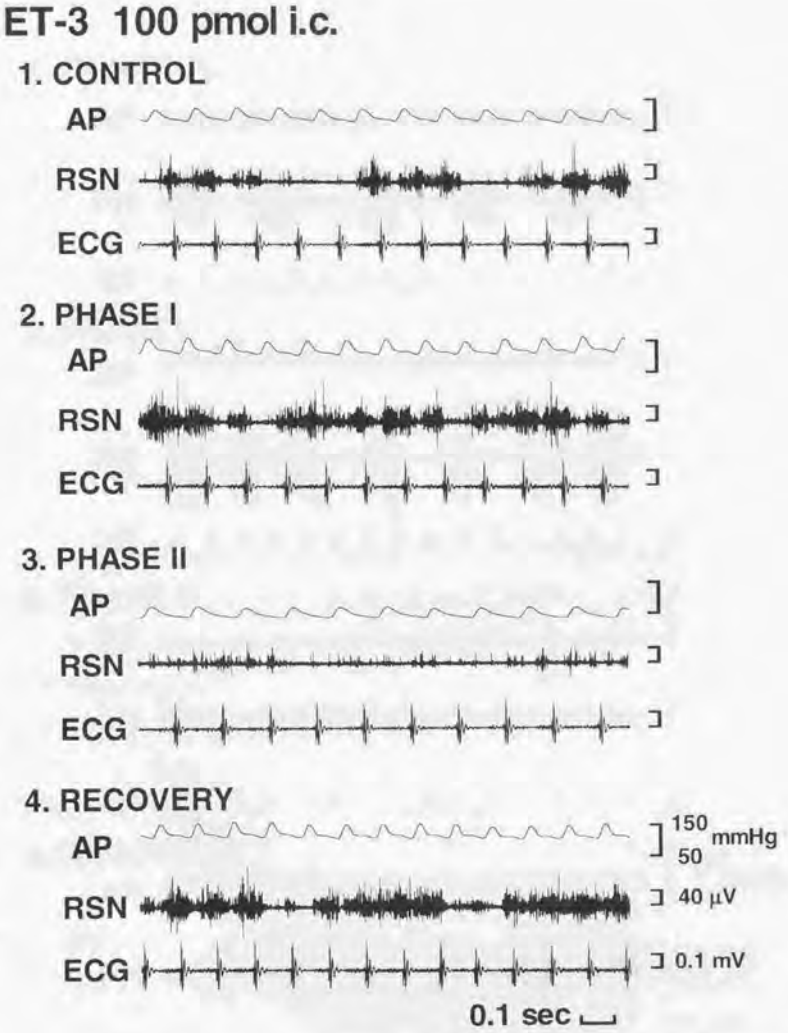


Figure 4C Effects on arterial pressure (AP) and renal sympathetic nerve discharges (RSN) of intracisternal administration of 100 pmol of ET-3 shown in enlarged time scale along with electrocardiogram (ECG).

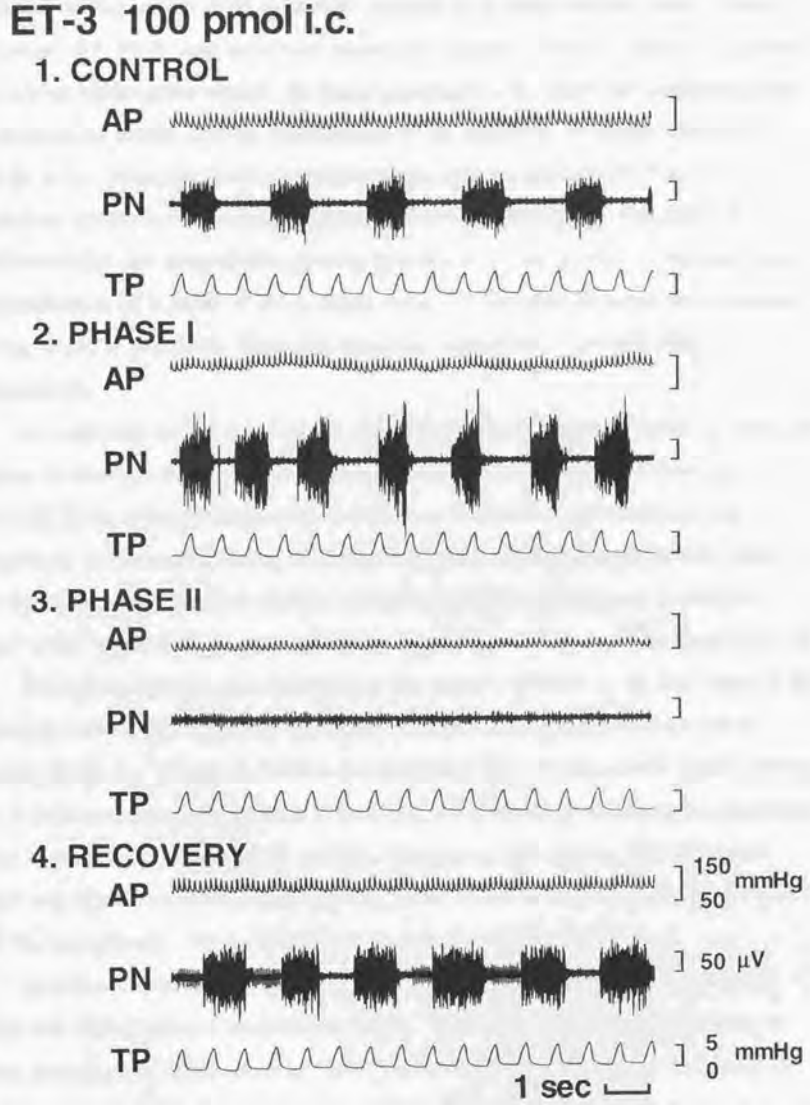


Figure 4D Effects on arterial pressure (AP) and phrenic nerve discharges (PN) of intracisternal administration of 100 pmol of ET-3 shown in enlarged time scale along with tracheal pressure (TP).

phase III during which these variables returned to or exceeded the control levels. However, AP, PNA, and burst rate sometimes remained below control throughout the 2-hour observation period. In those experiments in which AP returned to the baseline level within 2 hours, the duration of the depressor response was 62 ± 7 min (n = 5). Thus, the most prominent result of intracisternal ET-1 is its inhibitory action on the central respiratory activity, although it also affected cardiovascular and sympathetic activity to a lesser extent (Table 1). Actually, on administration of 1 pmol of ET-1, mean values of the peak decrease from control during phase II in RSNA, PNA and burst rate were 43%, 70% and 80%, respectively.

An injection of 100 pmol of ET-3 into the cisterna magna resulted in a similar pattern of changes in AP, RSNA, HR, PNA and burst rate to that following 1 or 10 pmol of ET-1, although there were individual variations in the duration and magnitude of responses among the animals (Figures 2, 3, 4C, and 4D and Table 1). On the other hand, 10 pmol of ET-3 elicited similar initial changes as with 0.1 pmol of ET-1 but there was no inhibitory period (phase II) with this dose of ET-3.

Following intracisternal injection of the vehicle (ACSF) in 10 rats, none of the variables exhibited statistically significant changes during the 2-h observation period (Table 1). However, there was a tendency that AP decreased slightly during the 2-h observation period, while RSNA and HR drifted toward the other direction. Two hours after injection of the vehicle, changed in AP, RSNA, HR, PNA and burst rate from baseline levels were -6 ± 5 %, 19 ± 8 %, 6 ± 4 %, -8 ± 5 %, and 1 ± 5 %, respectively. These changes were statistically insignificant.

Summarized in Table 1 are peak increases in MAP, RSNA, HR, PNA and burst rate during phase I (denoted as "Max." in the table), and peak decreases in them during phase II (denoted as "Min." in the table) in response to intracisternal administration of ET-1 and ET-3. At the dose of 0.1 pmol of ET-1 or 10 pmol of

TABLE 1. Peak changes in cardiorespiratory variables induced by intracisternal administration of ET-1 and ET-3 in urethane-anesthetized rats or in unanesthetized precollicular decerebrated rats

Drug	Dose (pmol)	MAP (mmHg) Control	Δ MAP Max.	Δ MAP Min.	RSNA (% of control) Control	Δ RSNA Max.	Δ RSNA Min.	HR (beats/min) Control	Δ HR Max.	Δ HR Min.	PNA (% of control) Control	Δ PNA Max.	Δ PNA Min.	Burst Rate (1/min) Control	Δ burst rate Max.	Δ burst rate Min.
Urethane-anesthetized rats																
Vehicle		90±3 (10)	3±2	-5±2	10±6 (5)	-2±6		449±16 (10)	6±3	-2±4	5±2 (5)	-1±1		48±6 (5)	1±1	-1±2
ET-1	0.1	99±3 (8)	10±3 **	-6±4	27±6 (6) **	-11±5		455±15 (8)	11±4 *	-2±7	10±2 (6)**	-20±8 *		40±3 (6)	3±1 *	-7±2 *
	1	102±6 (6)	13±6	-49±6 **	43±21 (6)	-43±8 **		467±15 (6)	13±4 *	-37±9 *	44±10 (6)**	-70±15 **		41±4 (6)	6±2 *	-33±4 **
	10	95±7 (9)	10±5	-53±6 **	42±21 (6)	-48±8 **		500±13 (9)	17±7 *	-69±16 **	27±10 (6)*	-79±13 **		40±2 (6)	3±1 *	-39±2 **
ET-3	10	96±3 (13)	13±2 **	-7±5	23±5 (10)**	-0±8		467±8 (13)	26±7 **	-0±8	33±9 (5)*	0±11		38±6 (5)	5±1 *	1±3
	100	90±2 (16)	17±3 **	-47±3 **	26±5 (12)**	-37±7 **		467±11 (16)	25±6 **	-58±9 **	72±14 (6)**	-98±1 **		36±2 (6)	12±4 *	-36±2 **
Unanesthetized precollicular decerebrated rats																
ET-1	1	88±10 (6)	18±7 *	-48±7 **	34±7 (5)**	-51±11 *		504±8 (6)	10±3 *	-53±19 *	65±14 (3)**	-83±10 *		31±3 (3)	3±2	-31±4 *
ET-3	100	101±5 (3)	28±10	-56±12 *	83±22 (2)	-53±15		531±7 (3)	23±5 *	-67±17	55±25 (3)	-72±12 *		47±8 (3)	5±2	-44±5 *

Values are means ± SE. Numbers in parentheses denotes experimental numbers. Abbreviations: ET-1, endothelin-1; ET-3, endothelin-3; HR, hart rate; MAP, mean arterial pressure; PNA, phrenic nerve activity; RSNA, renal sympathetic nerve activity. Δ denotes change in each variable from control. Significance of difference from the preinjection control value is denoted: * = P<0.05; ** = P<0.01 (paired t-test).

ET-3, peak increases in all the variables from control were statistically significant. Peak decreases in PNA and burst rate were also statistically significant in the case of 0.1 pmol of ET-1 but not in 10 pmol of ET-3. At the dose of 1 or 10 pmol of ET-1 or 100 pmol of ET-3, peak decreases in the five variables from control were all statistically significant. Although 100 pmol of ET-3 elicited statistically significant increases in all the measured variables, the initial increase in MAP and RSNA were not statistically significant in the case of 1 and 10 pmol of ET-1. At these doses of ET-1 in 15 animals, initial increases in RSNA and HR were observed in all the animals, whereas that of MAP occurred in all but three cases. In these cases, phase I was terminated by sudden and drastic decreases in these variables (Figure 3). Since the time of initiation of phase II following the drug administration varied considerably among the rats (range 2 - 19 min; n = 15), individual variations of peak increases in these variables was greater than those at the dose of 0.1 pmol of ET-1 or 10 or 100 pmol of ET-3. Consequently, peak increases in MAP and RSNA at these higher doses of ET-1 were statistically insignificant, although their mean values were comparable to or even greater than those at lower dose of ET-1.

In order to examine whether intracisternally administered ET might have reached the peripheral circulation and acted directly of the cardiovascular system, 100 pmol of ET-3 was intravenously injected as a bolus. The injection resulted, as observed in conscious rats [Yanagisawa, *et al.*, 1988], in an immediate fall in AP, lasting for less than 1 min, by about 10 - 20 mmHg below control, followed by a pressor response, lasting for 15 - 20 min, by about 20 - 30 mmHg above the pre-injection level. At a dose of 10 pmol of ET-3, a bolus intravenous injection did not produce any appreciable cardiovascular changes. Thus, intracisternally administered ET-3 caused responses quite different, with respect to their temporal pattern, magnitude, and the threshold dose, from its direct cardiovascular effects.

The brain surface stained with Evans Blue simultaneously injected with ET usually extended to the dorsal, lateral and ventral aspects of the brainstem, the ventral aspect of the cerebrum and the upper segments of the spinal cord. The surface of the cerebroventricular space including the floor of the fourth ventricle was not visibly stained.

These results may be summarized as follows. (1) Intracisternal ET-1 and ET-3 elicit both a short-latency pressor response with sympathoexcitation, tachycardia, and respiratory acceleration and a subsequent depressor response with sympathoinhibition, bradycardia, and respiratory inhibition. (2) The pressor response with sympathoexcitation, tachycardia, and respiratory acceleration was evoked at doses smaller than that required for the depressor response with sympathoinhibition, bradycardia, and respiratory inhibition. (3) Respiratory inhibition was striking in response to ET-1. (4) These changes were centrally mediated. Thus, centrally administered ET-1 and ET-3 affects tonic control of AP, RSNA, HR, PNA, and burst rate by the CNS.

Effect of precollicular decerebration and various general anesthetics on the response to ET-1 and ET-3

In order to examine whether cardiorespiratory responses to intracisternally administered ET were seriously affected by urethane, the same experiments were repeated in 9 unanesthetized precollicular decerebrated rats. One pmol of intracisternally administered ET-1 (Figure 5) or 100 pmol of ET-3 (Figure 6) resulted in a pattern of cardiorespiratory responses similar in its time course (Figures 1 ~ 3) and comparable in the magnitude of peak changes (Table 1) to those in urethane-anesthetized animals. Actually, between unanesthetized precollicular decerebrated and urethane-anesthetized rats, there was no statistically significant

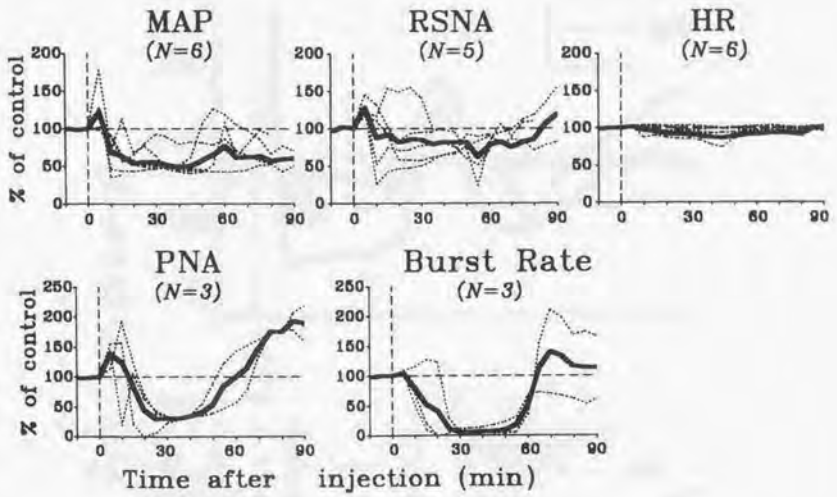


Figure 5 Effect of intracisternal administration of 1 pmol of ET-1 in unanesthetized precollicular decerebrated rats on MAP, RSNA, HR, PNA, and burst rate of PNA. Responses observed in individual animals are indicated by dotted lines and the average values are represented by heavy lines. Data are expressed as percent of preinjection control values.

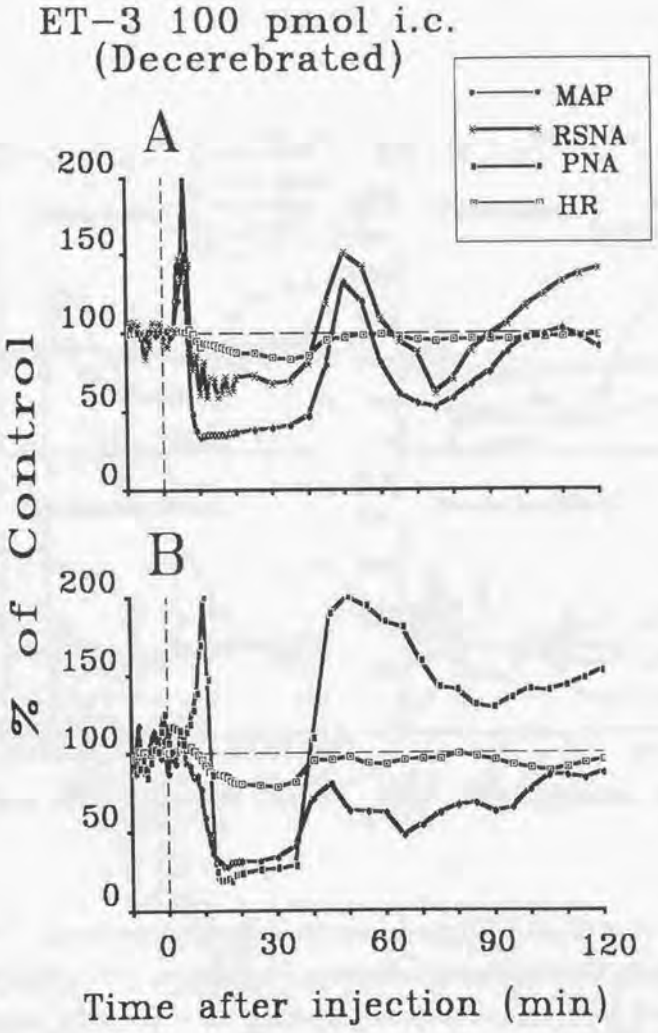


Figure 6 Cardiorespiratory effect of intracisternal administration of 100 pmol of ET-3 in unanesthetized precollicular decerebrated rats. Mean arterial pressure (MAP) and heart rate (HR) were recorded in both cases, whereas renal sympathetic nerve activity (RSNA) was recorded in A, and phrenic nerve activity (PNA) was recorded in B only. Data are expressed as percent of preinjection control values.

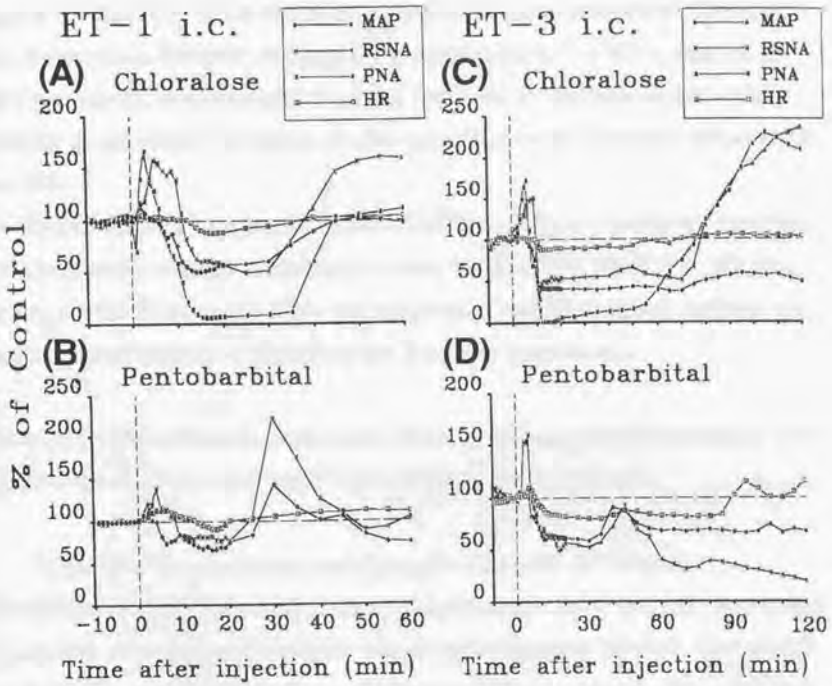


Figure 7 Responses to intracisternally administered ET-1 (A, B) or ET-3 (C, D) on MAP,RSNA, PNA, and HR in rats anesthetized by intraperitoneal injection of a-chloralose (100 mg/kg, A and C) or sodium pentobarbital (60 mg/kg, B and D). Each A - D represents results from different animals. Doses of intracisternal ET-1 were 1 pmol in A and 100 pmol in B, and that of ET-3 were 100 pmol in C and 1,000 pmol in D.

difference in peak changes of each cardiorespiratory variable induced by intracisternal injection of 1 pmol of ET-1 or 100 pmol of ET-3. A similar pattern of responses was elicited under chloralose anesthesia (100 mg/kg, i.p., initial dose; Figures 7A and 7C). When the rat was anesthetized by pentobarbital (60 mg/kg, i.p., initial dose), however, the dose of intracisternal ET-1 or ET-3 required to elicit comparable cardiovascular responses had to be 5 - 10 times of that under urethane or chloralose anesthesia, or after precollicular decerebration (Figures 7B and 7D).

In conclusion it appeared that urethane did not seriously distort the pattern of cardiorespiratory changes in response to intracisternal ET-1 and ET-3. For this reason, and for its relatively stable and long-lasting anesthetic effect, urethane was used as general anesthesia throughout the following experiments.

Effects of ganglion blockade, angiotensin II antagonist, vasopressin antagonist, and β-adrenergic blocking agent on ET-induced cardiovascular changes

To examine the mechanism underlying the change in AP induced by intracisternally administered ET, peripheral sympathetic nerve activity, angiotensin II receptors, or vasopressin receptors was pharmacologically blocked. The change in AP, RSNA, and HR induced by intracisternal ET-3 were totally eliminated by a continuous infusion of hexamethonium chloride (Figure 8). Thus, the ET-induced response of AP was mediated by the sympathetic nervous system. Furthermore, since vagal control of HR was already suppressed as a result of vagolytic action of gallamine triethiodide continuously infused throughout the experiment [Son and Waud, 1978], absence of the HR change after ganglion blockade in the present experiments implied that it was also mediated by the sympathetic nerve.

On the other hand, pretreatment with arginine vasopressin antagonist (n = 4)

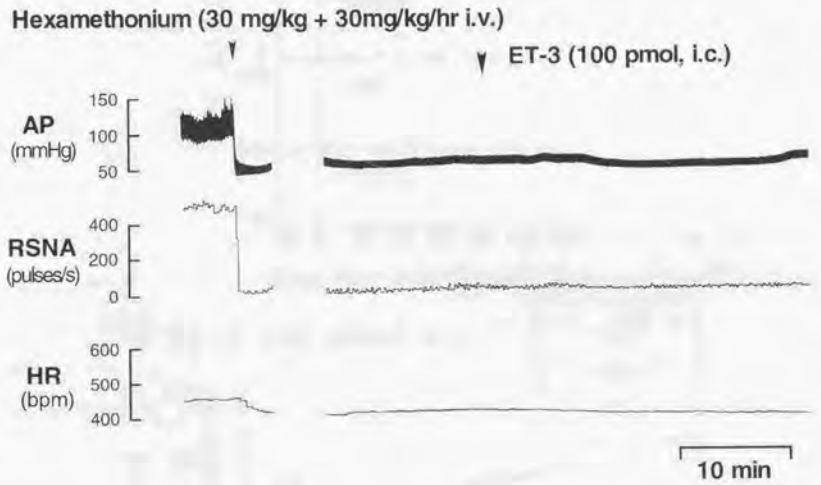


Figure 8 Effects of ganglion blockade on cardiovascular and sympathetic responses to ET-3 (100 pmol, i.c.). Hexamethonium chloride was injected at a dose of 30 mg/kg (i.v.) initially at the moment marked by the first arrow and continuously given at the rate of 30 mg/(kg·h) thereafter. Note that responses to administration of ET-3, marked by the second arrowhead, were abolished by pretreatment with ganglion blockade.

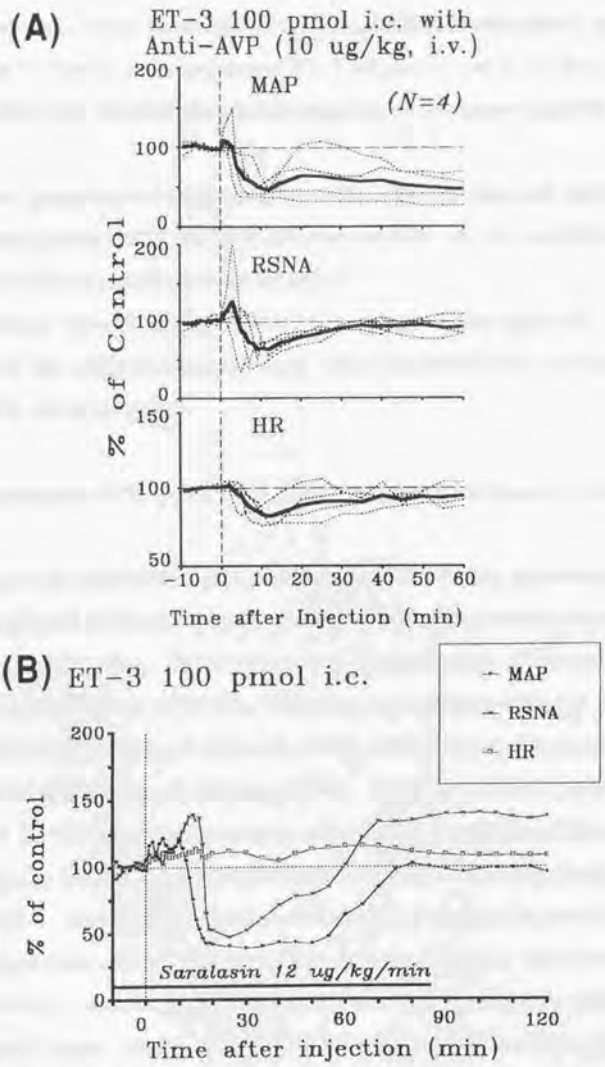


Figure 9 Responses to intracisternally administered 100 pmol of ET-3 on MAP, RSNA, and HR in rats pretreated with arginine vasopressin antagonist (Manning compound, 10 mg/kg, i.v.) in A or continuously infused with angiotensin II antagonist, salarasin (12 mg/(kg·min), i.v.) in B. Salarasin was infused during the period indicated by the horizontal line.

or salarasin, an angiotensin II antagonist (n = 1), did not conspicuously alter cardiovascular responses to intracisternal ET-3 (Figure 9), although these antagonists effectively blocked the pressor response to respective agonists (see method).

Moreover, pretreatment with the β-blocking agent of atenolol, propranolol, or timolol did not seriously alter the ET-3-induced response of AP and RSNA, though the response of HR was almost totally abolished.

In conclusion the efferent mechanism responsible, either primarily or exclusively, for the effect of intracisternally administered ET-3 on AP and HR was the sympathetic nervous system.

Effects of intracisternal ET-1 and ET-3 on the arterial baroreceptor reflex

Three types of experiments disclosed that intracisternally administered ET-1 (1 and 10 pmol) and ET-3 (100 pmol) modulated the cardiovascular control by the arterial baroreceptor reflex. In the first group of experiment in 40 rats, the cardiac-related fluctuations of RSNA, reflecting baroreceptor-initiated sympathoinhibition [Gebber and Barman, 1980], were compared before and after intracisternal administration of ET-1 and ET-3. An injection of 0.1 pmol of ET-1 or 10 pmol of ET-3 did not conspicuously affected the amplitude of the cardiac-related changes in rectified renal sympathetic discharges. Actually, in the case of 0.1 pmol of ET-1, the amplitude of the cardiac-related changes relative to the pre-injection control during the former and latter halves of phase I were 101 ± 5% and 102 ± 7% (n = 6), respectively. These values were not significantly different from the pre-injection value. At the dose of 1 or 10 pmol of ET-1 or 100 pmol of ET-3, their amplitude was conspicuously reduced during the latter half of phase I (Figures 10A, b and 10B, b). At the dose of 1 pmol of ET-1, the relative amplitude of the

cardiac-related changes during the former and latter halves of phase I was 106 ± 5% and 41 ± 9% (n = 6), respectively. The corresponding values at the dose of 10 pmol of ET-1 were 110 ± 6% and 32 ± 4% (n = 6), respectively. At both doses, the values during the former half of phase I were not significantly different from pre-injection values, whereas those during the latter half were significantly smaller ($P < 0.01$). Throughout the phase I, MAP remained above the threshold pressure of the arterial baroreceptor reflex (78±9 mmHg in normotensive rats according to Brown *et al.* [Brown, *et al.*, 1976]). During phase II following intracisternal administration of 1 or 10 pmol of ET-1 or 100 pmol of ET-3, the cardiac-related changes were not detected because MAP decreased below the threshold pressure of the arterial baroreceptor reflex (Figures 10A, c and 10B, c). The arterial baroreceptor reflex was restored during phase III (Figures 10A, d and 10B, d).

In the second group of experiments in 12 rats, reflex changes in RSNA induced by bolus intravenous injections of phenylephrine at different doses were plotted against alterations in MAP. When such data were pooled from three rats to which 1 pmol of ET-1 was administered, the slope of the regression line, reflecting the gain of the arterial baroreceptor reflex, was -0.22 $mmHg^{-1}$ during phase II as against -0.95 $mmHg^{-1}$ during the pre-injection period (Figure 11A). The difference was statistically significant ($p<0.01$). Likewise, with respect to the pooled data from four rats which were given 10 pmol of ET-1, the corresponding values were -0.26 $mmHg^{-1}$ and -0.90 $mmHg^{-1}$, respectively. The difference was statistically significant ($p<0.01$). Virtually the same result obtained for 100 pmol of ET-3 (Figure 11B). Corresponding values for ET-3 were -0.22 $mmHg^{-1}$ and -0.97 $mmHg^{-1}$, respectively. During phase III, the reflex restored partially as evidenced by return of the slope value (-0.52 $mmHg^{-1}$) towards the value during pre-injection period.

In one rat that received 100 pmol of ET-3 intracisternally, the aortic nerve

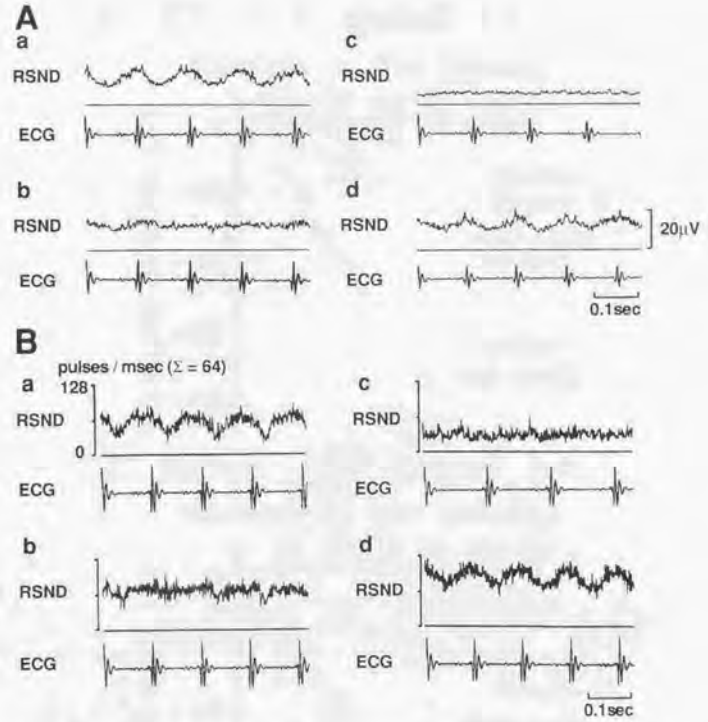


Figure 10 Inhibition of the arterial baroreceptor reflex by intracisternal administration of 10 pmol of ET-1 in A or 100 pmol of ET-3 in B. In each tracing (a-d), cardiac-related fluctuations of rectified renal sympathetic nerve discharges (RSND), obtained as the post R-wave time histogram over 32 or 64 successive sweeps, were determined during the control period (a; MAP = 90 ~ 100 mmHg), latter part of phase I (b; MAP = 120 ~ 130 mmHg), phase II (c; MAP = ~50 mmHg) and phase III (d; MAP = 90 ~ 100 mmHg) and shown along with electrocardiogram (ECG). In a, b, and d, MAP remained within the operating range of the arterial baroreceptor reflex. Then, the amplitude of fluctuations represented effectiveness of the arterial baroreceptor reflex. Note that the reflex was already inhibited at the latter period of phase I (b) but fully recovered at phase III (d). In A, RSND is expressed as the average of the nerve discharges, whereas in B, it is expressed as the pulse counts of unitary transformed nerve activities.

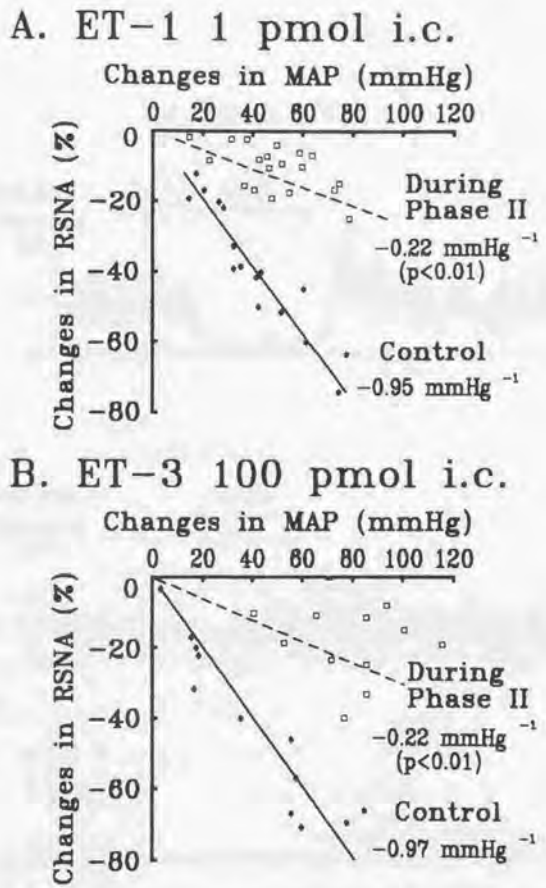


Figure 11 Inhibition of the arterial baroreceptor reflex by intracisternal administration of 1 pmol of ET-1 in A or 100 pmol of ET-3 in B. The relationship between the reflex changes in RSNA and alterations of MAP induced by graded bolus injection of phenylephrine, both expressed as the change from pre-injection level, was obtained during the control period (solid line) and phase II (broken line). Data points were collected from 4 experiments in A and 5 experiments in B. Slope of the regression lines, indicating the gain of arterial baroreceptor reflex, was significantly reduced during phase II as compared to those of control period (analysis of covariance) in both cases of ET-1 and ET-3.

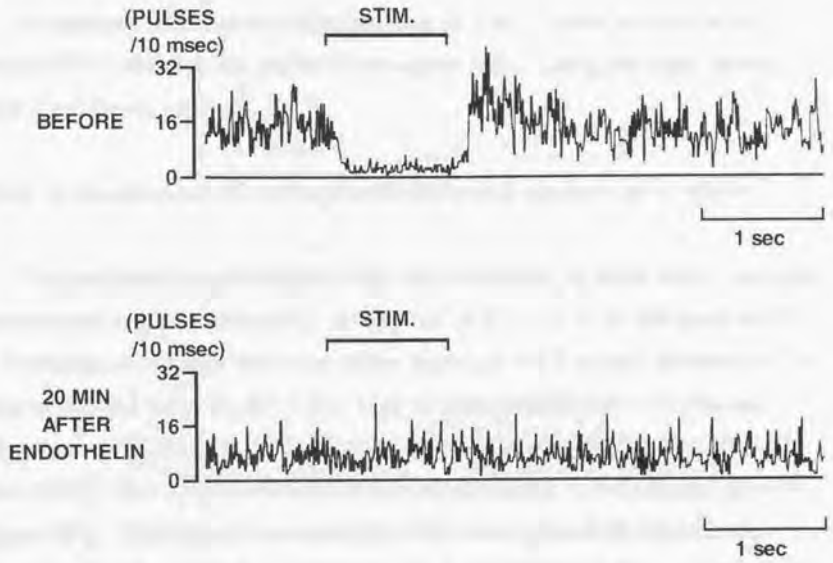


Figure 12 Inhibition of the arterial baroreceptor reflex by intracisternal administration of 100 pmol of ET-3 as evidenced by attenuation of reflex inhibition in renal sympathetic nerve discharges to electrical stimulation of aortic nerve. Peri-stimulus time histogram was constructed before (upper panel) and after (lower panel, in phase II) administration of ET-3 by counting standard pulses obtained from window discriminator fed with left renal sympathetic nerve discharges for successive 16 stimulations (10 V, 100 Hz, pulse width 0.5 ms, for 1s) applied to the right aortic nerve at the time indicated by horizontal bars.

was stimulated to elicit arterial baroreceptor reflex. As could be seen in the peri-stimulus time histogram (Figure 12), reflex inhibition of rectified renal sympathetic nerve discharge was much weaker during phase II as compared to the pre-injection period.

In summary intracisternal administration of 1 or 10 pmol of ET-1 or 100 pmol of ET-3 inhibited the arterial baroreceptor reflex during the latter half of phase I and during phase II.

Effects of intracisternal ET on peripheral and central chemoreceptor reflexes

The peripheral chemoreceptor reflex was induced by hypoxia before and after intracisternal administration of 0.1 or 10 pmol of ET-1 or 10 or 100 pmol of ET-3. Inhalation of nitrogen for 1 min before injection of ET or after injection of low doses of ET (0.1 pmol for ET-1 (n = 1) or 10 pmol for ET-3 (n = 1)) elicited reflex increase in PNA. By contrast, injection of 10 pmol of ET-1 (n = 1) or 100 pmol of ET-3 (n = 1) almost abolished the reflex increase in PNA during phase II (Figure 13). The central chemoreceptor reflex was induced by hypercapnia. Inhalation of 5 % carbon dioxide for 3 min before injection of ET elicited definite increases in PNA and burst rate. Injection of 1 pmol of ET-1 (n = 2) or 100 pmol of ET-3 (n = 2) completely diminished the reflex increase in PNA or burst rate during phase II (Figure 14).

In summary, intracisternally administered ET-1 (more than 1 pmol) or ET-3 (100 pmol) inhibited both peripheral and central chemoreceptor reflexes during phase II.

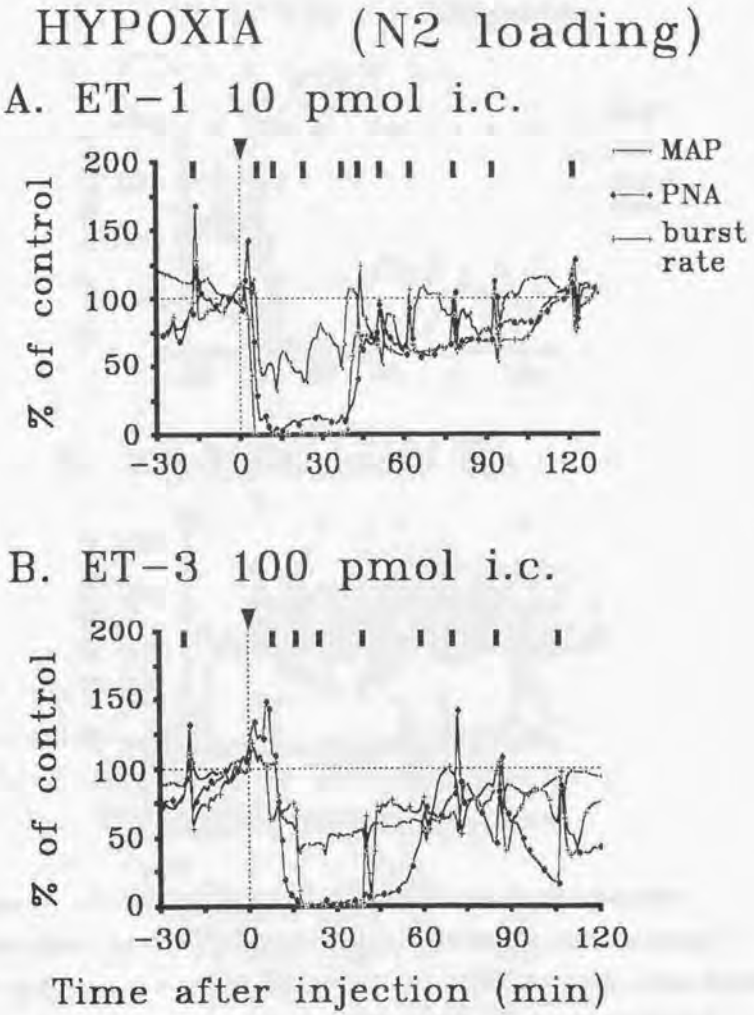


Figure 13 Inhibition of peripheral chemoreceptor reflex by intracisternal administered 10 pmol of ET-1 (A) or 100 pmol of ET-3 (B). Reflex increases in PNA and burst rate induced by hypoxia with inhalation of 1:1 mixture of the room air and 100 % nitrogen applied during the periods indicated by horizontal bars were attenuated or even completely abolished during phase II. Data are expressed as percent of preinjection control values.

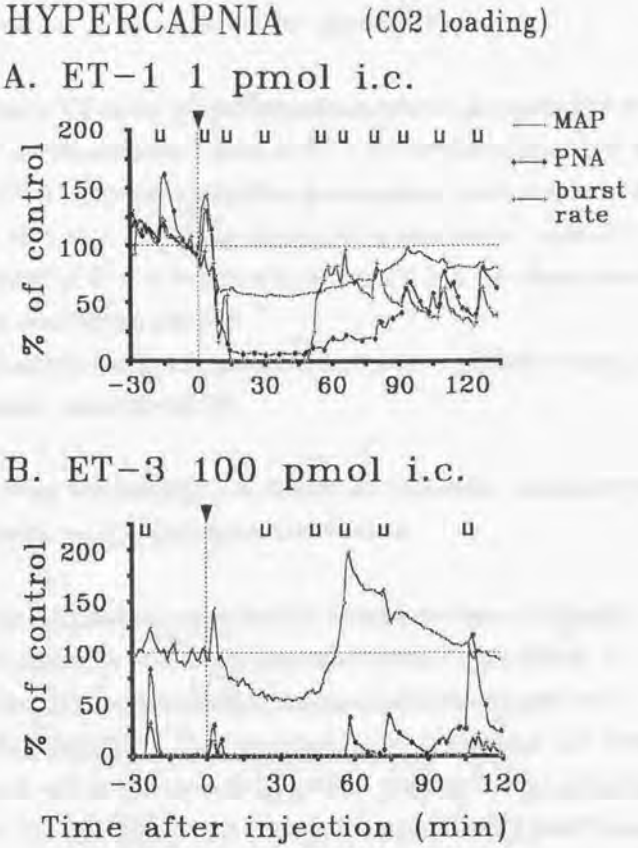


Figure 14 Inhibition of central chemoreceptor reflex by intracisternally administered 1 pmol of ET-1 (A) or 100 pmol of ET-3 (B). Reflex increases in PNA and burst rate induced by hypercapnia with inhalation of 5 % carbon dioxide applied during the periods indicated by hollow-shaped bars were diminished during phase II. A and B were representative data from two animals, and similar results were obtained other two animals. In the case of B, the animal was ventilated with 100 % oxygen during entire period except CO_2 inhalation periods so that reflex increase in PNA and burst rate could be easily seen. Data are expressed as percent of preinjection control values.

Effects of intracisternal ET on the somato-sympathetic reflex

The effect of ET on the somato-sympathetic reflex was examined in 10 rats. Intracisternal administration of 1 pmol of ET-1 (n = 5) slightly weaken or had virtually no effect on the reflex excitation in sympathetic nerve activity in response to activation of A- or A and C- fiber groups of the sural nerve (Figure 15). By contrast, 10 pmol of ET-1 (n = 5) or 100 pmol of ET-3 (n = 1) almost completely abolished the reflex during phase II.

In summary the somato-sympathetic reflex was also inhibited during phase II by intracisternally administered ET.

6.2 Brain Sites Responsible for Effects of Centrally Administered Endothelin on Cardiorespiratory Control

Brain sites (CNS sites) responsible for modulatory action of centrally administered endothelin were sought in the next series of experiments. As explained above, ET-induced changes were preserved after precollicular decerebration. Furthermore, the arterial baroreceptor, peripheral and central chemoreceptor, and somato-sympathetic reflexes were inhibited by intracisternally administered ET. Therefore, major CNS sites responsive to ET must be located below the midbrain and along the reflex pathway subserving these reflexes.

Effects of topical application of ET to the NTS and area postrema on central cardiorespiratory control

A unilateral injection of 0.1 - 10 pmol of ET-1 or ET-3 into the NTS at the level of the calamus scriptorius resulted in increases in AP, RSNA, HR, PNA, and

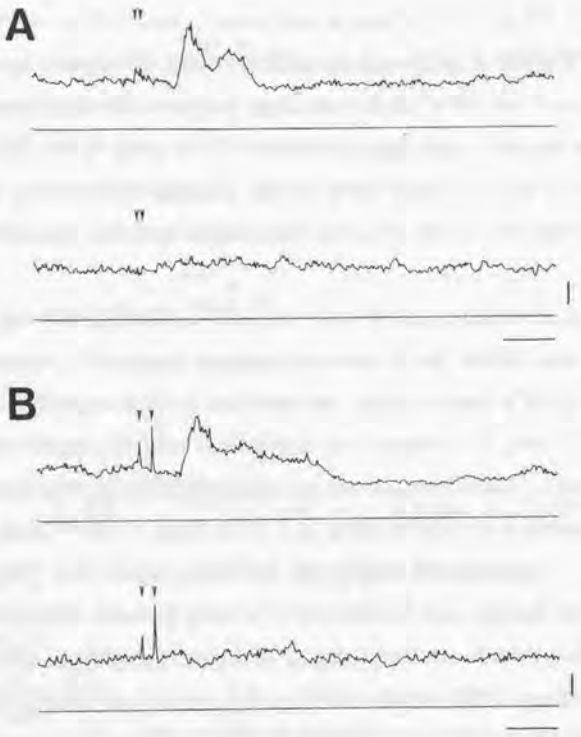


Figure 15 Inhibition of somato-sympathetic reflex by intracisternal administered 10 pmol of ET-1. Peri-stimulus time histogram of renal sympathetic nerve discharges (RSND) was constructed before (upper traces) and after (lower traces, in phase II) administration of ET-1 by averaging rectified RSND for successive 32 stimulations applied to the left sural nerve. In the panel A, two pulses of negative rectangular pulse with 5 V, 100 Hz (pulse duration 0.2 ms) were applied at the time indicated by arrowhead to activate group II and group III fibers, wheras in B, stimulation intensity was 30 V and frequency was 40 Hz (two pulses of 0.5 ms duration) to activate groups II ~ IV fibers. Vertical and horizontal calibrations are 0.1 mV and 0.1 s, respectively. Representative records from an animal were shown and similar results were obtained from other four animals.

burst rate (Figure 16 and Table 2). The response occurred within 1 min and lasted for more than 30 min at the doses of more than 4 pmol of ET-1 or ET-3. There were no subsequent decreases in these variables corresponding to phase II as was seen in intracisternal injection or topical application to the VSM (see Section 5-1 and below). ACSF or 0.01 pmol of ET-1 elicited no significant changes in cardiorespiratory parameters examined. On the other hand, 0.1 ~ 10 pmols of ET-1 and ET-3 significantly and dose-dependently increased all the measured variables (Table 2).

When injected into the rostral NTS at the level 2 mm rostral to the calamus scriptorius, 4 pmol of ET-1 caused sustained increases in AP, RSNA, and HR without significant changes in PNA and burst rate, while 4 pmol of ET-3 did not cause any notable changes in these cardiorespiratory variables (Figure 17). Rostrocaudal distribution of simultaneously injected dye was about 1.0 mm centered at the injection point. When 4 pmol of ET-1 (n = 10) or ET-3 (n = 6) was injected within the medulla 1 mm lateral to the NTS, no changes were elicited.

On the other hand, when 10 pmol of ET-1 or ET-3 was injected into the area postrema in another 10 rats, the changes in cardiorespiratory variables were much small in size and opposite in direction to those following the NTS injection (Figure 17). When 10 pmol of ET-1 or ET-3 (n = 3, for each) was injected into the vertebral artery, no conspicuous changes in cardiorespiratory parameters was observed (Figure 18). In the latter series of experiments, subsequently injected dye reached the area postrema. Thus, most effective site for above mentioned effect of ET was restricted to the intermediate and caudal portions of the NTS.

Although ET-1 and ET-3 elicited cardiorespiratory changes over a similar dose range, there were some differences in the magnitude of cardiorespiratory changes (Table 2). Namely, the peak change in MAP and RSNA in response to 4 pmol of ET-3 was significantly greater than those in response to the same dose of

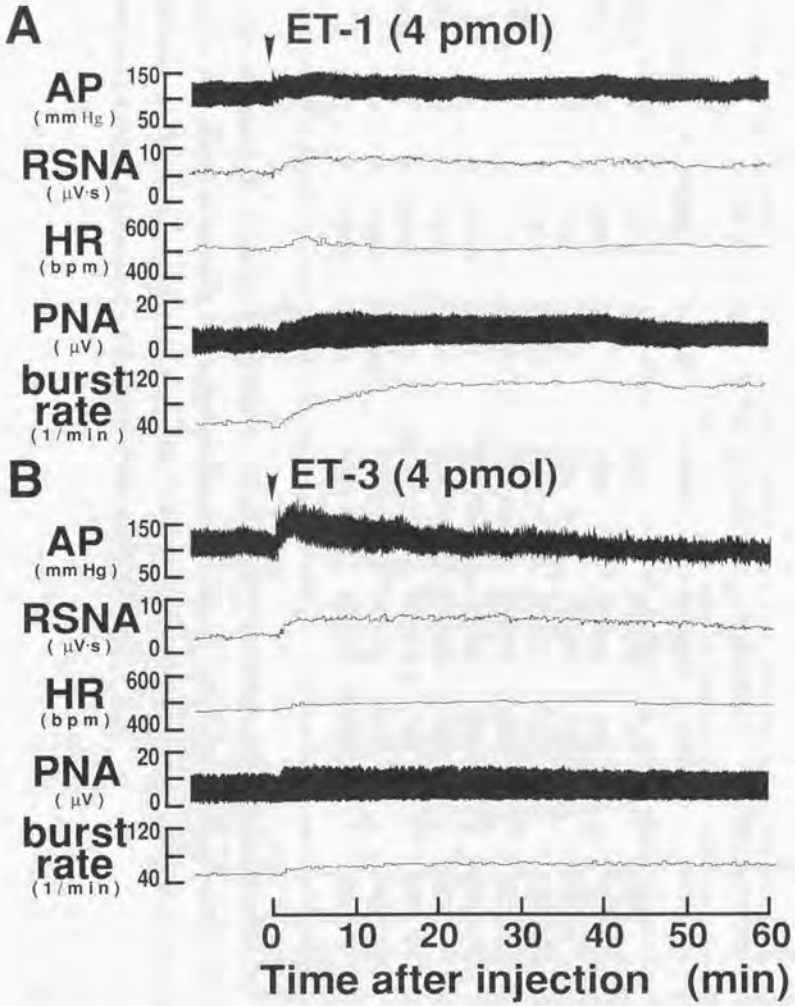
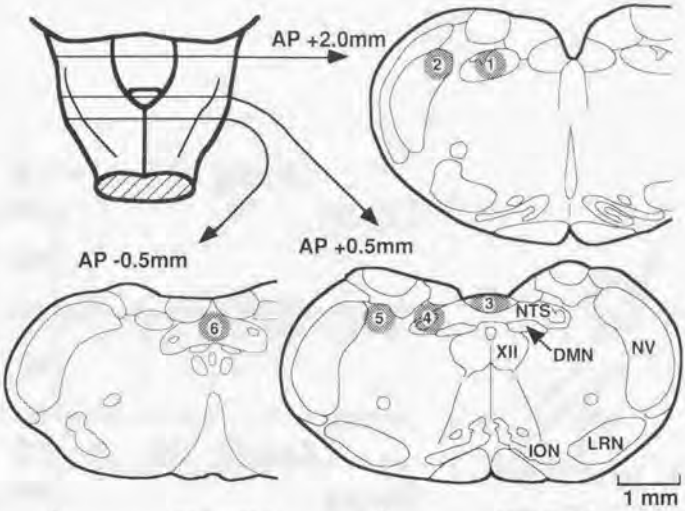


Figure 16 Polygraph records illustrating responses of arterial pressure (AP), integrated renal sympathetic nerve activity (RSNA), heart rate (HR), phrenic nerve activity (PNA), and burst rate of PNA to microinjection of 4 pmol of ET-1 (A) or ET-3 (B) in 0.2 μl of arterial cerebrospinal fluid into the intermediate region of the NTS. ETs were applied at the moment marked by arrowheads. Note that only excitation was seen in all the variables.

TABLE 2. Peak increases in cardiorespiratory variables induced by microinjection of ET-1 and ET-3 to the intermediate region of the NTS

Drug	Dose (pmol)	n	MAP (mmHg) Control	Δ MAP	RSNA (% of control) Δ RSNA	HR (beats/min) Control	Δ HR	PNA (% of control) Δ PNA	Burst Rate (1/min) Control	Δ burst rate
Vehicle		6	91±4	2±2	10±5	477±13	4±3	5±6	45±4	0±4
ET-1	0.01	6	97±6	9±4	14±6	468±25	6±3	22±10	44±3	7±6
	0.1	6	94±6	14±3 **	15±4 *	488±19	10±3 *	54±21 *	48±4	11±4 *
	1	6	89±4	13±2 **	25±5 **	473±23	13±4 *	51±17 *	43±3	7±2 *
	4	6	107±8	11±3 **	20±5 *	480±12	12±3 **	82±12 **	48±2	15±5 *
	10	8	88±5	17±5 *	39±15 *	499±12	28±8 *	116±28 **	44±4	41±9 **
ET-3	0.1	7	101±5	11±2 **	21±4 **	488±10	12±3 *	18±4 **	51±5	8±3 *
	1	8	113±4	13±3 **	24±5 **	476±13	7±3 *	25±7 **	43±5	9±2 **
	4	7	94±5	25±3 **##	80±18 **#	473±11	21±3 **#	55±19 *	46±3	10±4 *
	10	7	92±4	18±5 *	67±21 *	477±12	17±6 *	52±11 **	52±3	8±3 *##

Values are means ± SE. n = number of experiments. Abbreviations: ET-1, endothelin-1; ET-3, endothelin-3; HR, hart rate; MAP, mean arterial pressure; PNA, phrenic nerve activity; RSNA, renal sympathetic nerve activity. Δ denotes change in each variable from control. Significance of difference from the preinjection control value is denoted: * = P<0.05; ** = P<0.01 (paired t-test). Significance of difference between the same dose group of ET-1 and ET-3 is denoted: # = P<0.05; ## = P<0.01 (un-paired t-test).



	Effect of ET-1						Effect of ET-3					
site #	n	ΔMAP (mmHg)	ΔRSNA (%)	ΔHR (bpm)	ΔPNA (%)	Δburst rate (1/min)	n	ΔMAP (mmHg)	ΔRSNA (%)	ΔHR (bpm)	ΔPNA (%)	Δburst rate (1/min)
1	6	20±4**	32±8**	28±6**	8±10	1±3	7	5±2	5±3	3±2	5±3	1±1
2	4	3±6	4±7	3±3	7±9	0±1	0					
3	6	-11±1**	-8±5	-9±4	-9±3	-11±3*	4	-18±5*	-23±5*	-18±6	-17±4*	-8±2
4	6	11±3**	20±5*	12±3**	82±12**	15±5*	7	25±3**	80±18**	21±3**	55±19*	10±4*
5	6	-1±2	-1±3	5±3	6±3	1±1	6	-1±1	3±3	3±2	3±6	-1±2
6	6	0±6	11±7	7±9	106±30*	24±4**	6	19±3**	38±12*	17±4*	48±13*	13±2**

Figure 17 Neuroanatomical site specificity of cardiorespiratory responses elicited by unilateral microinjection of ET-1 or ET-3 (both in 200 nl of artificial cerebrospinal fluid) into six sites within the medulla oblongata; into rostral, intermediate, caudal commissural regions of the NTS, area postrema, and into two other sites, each 1 mm laterally apart from the two NTS sites. Injection sites from 1 to 6 are shown diagramatically. Injected doses were 10 pmol in area postrema and 4 pmol in other five sites. Totally 64 injections were made in 40 rats. Values are mean ± S.E. Δ denotes changes in each variable from control. Significance of difference from the preinjection control value is denoted: * = $p<0.05$; ** = $p<0.01$ (paired t-test). Abbreviations in this and figure 19 are; DMN, dorsal motor nucleus of vagus nerve; ION, inferior olivary nucleus; LRN, lateral reticular nucleus; NTS, nucleus tractus solitarius; NV, spinal nucleus of trigeminal nerve; XII, nucleus of hypoglossal nerve.

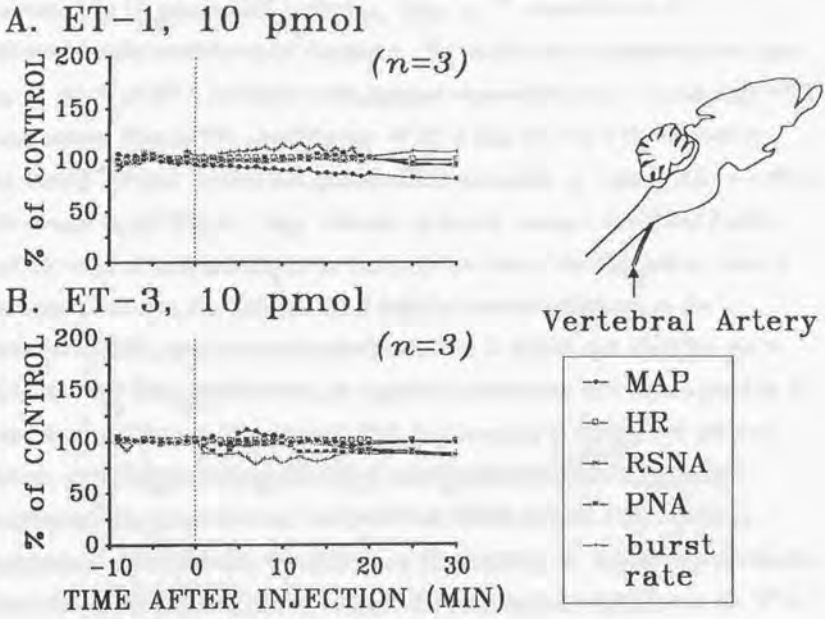


Figure 18 Effects of bolus injection of 10 pmol of ET-1 (A, n=3) and ET-3 (B, n=3) into the vertebral artery. Curves represent mean values and vertical bars are S.E. ETs dissolved in 10 µl of arterial cerebrospinal fluid were injected.

ET-1. On the contrary, the peak change in burst rate in response to 10 pmol of ET-1 was statistically greater than that in response to the same dose of ET-3. Moreover, the peak change in PNA in 10 pmol of ET-1 tended to be greater than that caused by 10 pmol of ET-3 ($P<0.1$). Thus, ET-3 seemed to elicit preferentially the cardiovascular responses. To confirm this hypothesis, the same dose (4 pmol) of ET-1 and ET-3 were injected sequentially into left and right NTS of one animal (Figure 19). An injection of ET-1 into the left NTS resulted in a long-lasting increase in PNA and burst rate and moderate increase in RSNA with a little change in AP (Figure 19A). Absence of drastic changes in AP and RSNA does not seem to be ascribable to an inappropriate site of microinjection, since a subsequent injection of 4 pmol of ET-3 into the corresponding site in the contralateral NTS resulted in substantial increases in RSNA and MAP but not in PNA and burst rate. Furthermore, in separate experiments in 2 rats, 4 pmol of ET-3 was first microinjected into the left NTS and increases in RSNA and AP were elicited. A subsequent injection of ET-1 into the contralateral NTS caused a conspicuous change in PNA but not in AP and RSNA (Figure 19B). In these experiments, simultaneously injected Evans Blue stained the area around the tractus solitarius usually covering dorsal, medial, and ventrolateral subnuclei of the NTS [Kalia, *et al.*, 1984].

In summary, microinjection of ET-1 and ET-3 to the NTS caused sustained increases in cardiorespiratory variables. Although ET-1 and ET-3 produced a similar pattern of changes and the effective dose range was comparable with the two isopeptides, their effect was not exactly identical. Topical application of ET to the area postrema resulted in small decreases in cardiovascular variables.

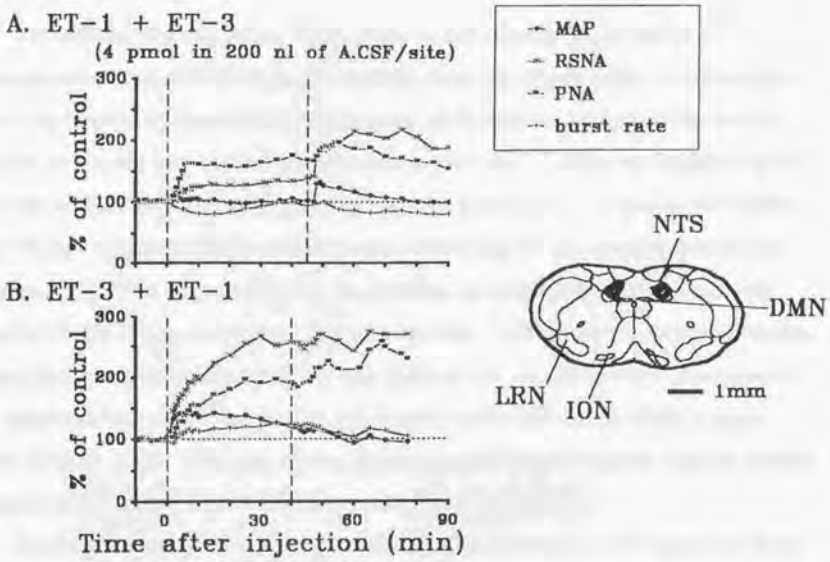


Figure 19 Comparison of effects of local microinjection of ET-1 and ET-3 into the NTS. In A, ET-1 was injected into the left NTS at the moment indicated by the vertical broken line. Forty-five min after that, ET-3 was injected into the right NTS. In B, ET-3 was injected into the left NTS first. Forty min after that, ET-1 was given to the contralateral NTS. The amount of injected ET was always 4 pmol in 200 nl of artificial cerebrospinal fluid. Changes from pre-injection values in MAP, RSNA and HR were expressed in percent and illustrated on the left panels. Injection sites in each experiment were shown by the shaded area on the frontal section of the medulla at the level of calamus scriptorius. For abbreviations, see figure 17.

Effects of topical application of ET to the VSM on central cardiorespiratory control

To examine the role of the VSM, ET was delivered to the VSM by a micropipette penetrated through the medulla from the dorsal surface or through a direct application to the VSM after exposure of the ventral surface of the lower brainstem. In the first type of experiments in five rats, a unilateral injection of ET-3 by the amount as small as 20 pmol elicited the pattern of responses in AP, RSNA, and HR that was remarkably similar to that following the intracisternal injection (Figure 20A). The area reached by the solution, as estimated by simultaneously injected Evans Blue, varied from animal to animal. Whenever the response similar to that following intracisternal ET-3 was elicited, the stained surface area covered the portion of the brainstem medial and rostral to the root of the XIIth cranial nerve (Figure 20B). This part of the medullary surface overlapped with the central chemosensitive area in the VSM [Bruce and Cherniack, 1987].

In the second type of experiments, the VSM was exposed and a piece of filter paper soaked with 1 pmol of ET-1 (n = 10) or 40 pmol of ET-3 (n = 6) dissolved in the ACSF was applied to the VSM rostral to the root of the hypoglossal nerve. This area corresponds approximately to the intermediate (or S) and caudal half of the rostral (or M) areas of the VSM [Trouth, *et al.*, 1973]. As expected, the pattern of changes in AP, HR, RSNA, PNA and burst rate were essentially identical to that following intracisternal administration (Figure 21). On the other hand, topical application of the filter paper soaked in artificial cerebrospinal fluid to the VSM in three rats did not produce appreciable changes in AP, RSNA, HR, PNA, or burst rate. Although the actual amount of ET released from the filter paper and acting on the VSM can not be determined, one can still say that topical application of ET to the VSM elicits the pattern of cardiovascular and sympathetic vasomotor changes

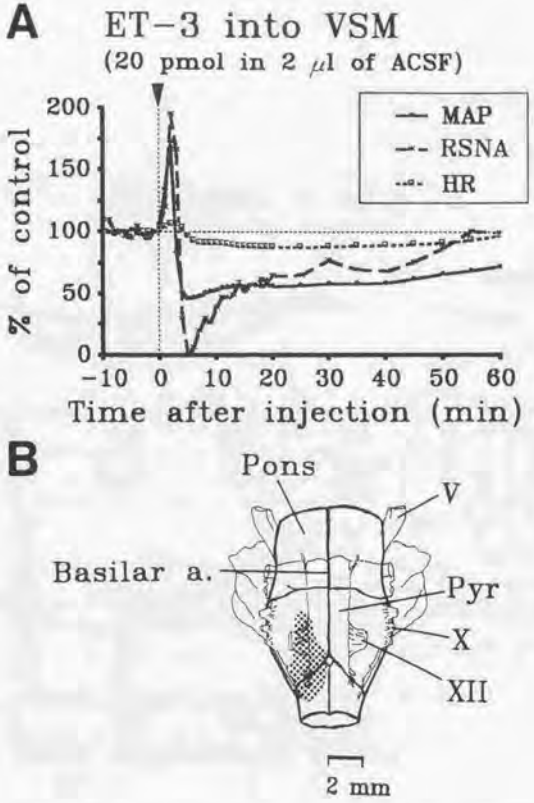


Figure 20 Typical example illustrating effects of topical application of ET-3 to the ventral surface of the medulla (VSM). A micropipette filled with ET-3 in arterial cerebrospinal fluid was penetrated at the dorsal surface of the medulla, advanced ventrally, passed through the brainstem, and placed in the subarachnoid space below the area of VSM. Responses to 20 pmol of ET-3, pressure injected in a volume of 2 μl, are illustrated in A. The extent of the brain surface directly reached by ET-3 was assessed by simultaneously injected Evans Blue (B). Note that the area reached by dye overlapped with the central chemosensitive area. Abbreviations in A and B are as follows: ACSF, artificial cerebrospinal fluid; Basilar a., basilar artery; Pyr, pyramidal tract; V, trigeminal nerve; X, vagus nerve; XII, hypoglossal nerve.

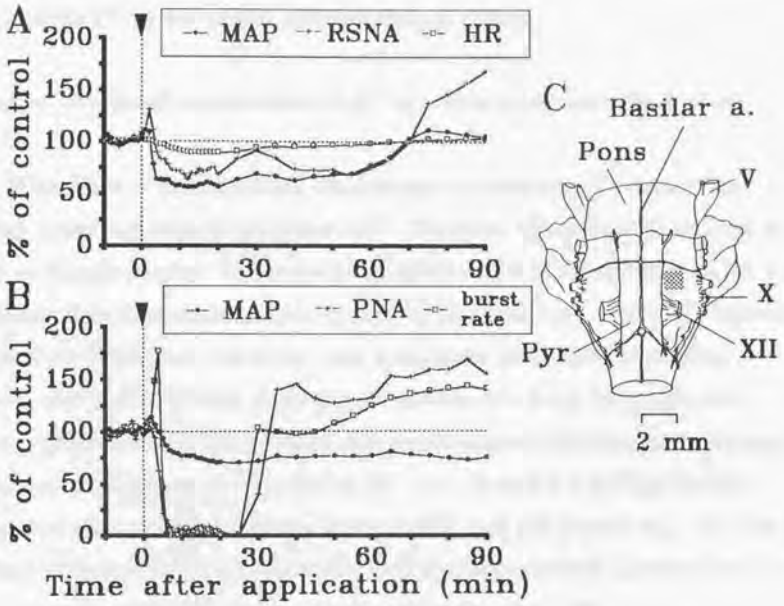


Figure 21 Effects of topical application of ET-1 to the ventral surface of the medulla (VSM) on MAP, RSNA and HR (A) or MAP, PNA and burst rate (B). Recordings in A and B were obtained from two different rats. A piece of filter paper (1 x 1 mm) soaked with 1 pmol of ET-1 was placed on the exposed VSM as shown by the dotted area in C which illustrates the ventral surface of the lower brainstem. For abbreviations, see figure 20.

similar to that following an intracisternal injection.

In summary topical application of ET-1 and ET-3 to the VSM resulted in the same pattern of cardiorespiratory changes as with intracisternal administration. It seems likely that the VSM is involved critically in the effect of intracisternally administered ET on the central cardiorespiratory control.

Effects of intrathecal administration of ET on central cardiovascular control

When ET was intracisternally administered, simultaneously injected dye stained upper segments of the spinal cord. Therefore, ET might have reached and acted on the spinal cord. To determine the effect of ET on the spinal cord, ET was administered to the thoraco-lumbar segment of the spinal cord. Since this segment included the distribution site of the renal sympathetic preganglionic neurons [Strack, *et al.*, 1988] but not of the phrenic neurons which lay in the cervical segment [Kuzuhara and Chou, 1980], only cardiovascular variables were measured. An intrathecal injection of 100 pmol of ET-1 (n = 3) or ET-3 (n = 3) into the spinal cord elicited modest changes in AP, RSNA, and HR (Figure 22). The pattern of these changes was different from that following intracisternal administration and the magnitude of the changes were much smaller than those following an intracisternal injection of ET. Simultaneously injected Evans Blue spread over 5 - 10 segments of both the dorsal and ventral surfaces of the spinal cord that encompassed thoracic and lumber segments. Specific segments of the spinal cord or the exact location within a spinal segment for the drug action was not determined.

In summary an intrathecal injection of a massive dose of ET resulted in modest cardiovascular changes much smaller than those following intracisternal administration.

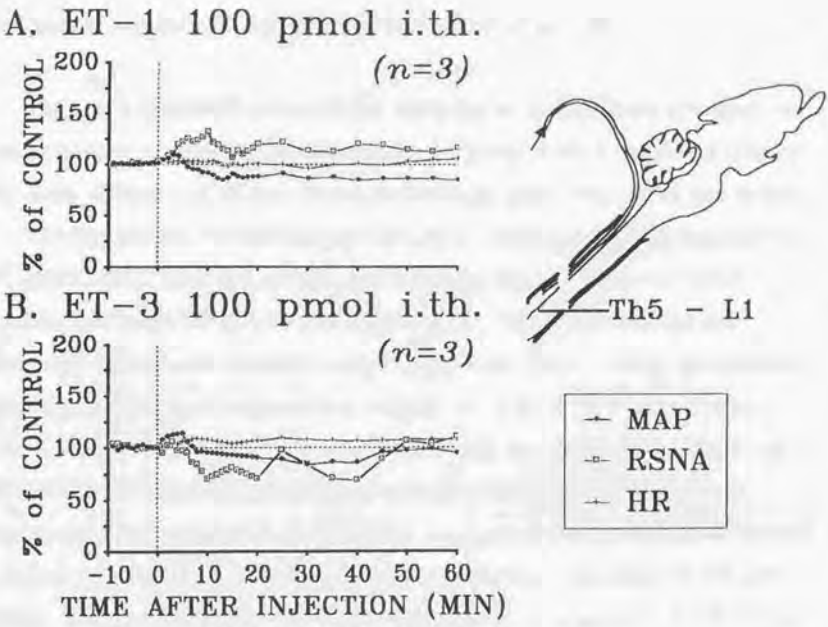


Figure 22 Effects of intrathecal administration of 100 pmol of ET-1 (A, n=3) and ET-3 (B, n=3). Curves represent mean values. ETs dissolved in 10 µl of arterial cerebrospinal fluid were injected spinal subarachnoid space at the level of Th5 - L1 (right panel).

6.3 Subregion of the Ventral Surface of the Medulla Responsible for Central Cardiorespiratory Effects of Endothelin

Two types of responses to topical application of ET to the VSM

Through a systematic survey of the VSM for cardiorespiratory changes, two distinct patterns of responses to application of 1 pmol of ET-1 was found (Figure 23). Each of them was elicited from a discrete subregion of the VSM (see below).

The first pattern, termed the type I response, consisted of initial increases in AP, RSNA, PNA, burst rate of PNA and HR followed by decreases in these variables that lasted for 20 - 60 min (Figure 23A). The initial increase and subsequent decrease are hereafter called, respectively, the excitatory and inhibitory components. The type I response was typically elicited by ET-1 delivered to a point 2.5 mm rostral and 2.0 mm lateral to the reference point (Figure 23A; see METHODS for definition of the reference point). The type II response was characterized by a marked decrease in PNA usually accompanied by an increase in burst rate (Figure 23B). Sometimes, there were moderate decreases in AP and RSNA. The type II response was produced typically by application of ET-1 to a point 0.0 mm rostral and 1.0 mm lateral to the reference point. The subregions of the VSM in which ET-1 produced the type I and II responses were termed the rostral and caudal ET-sensitive areas, respectively.

Application of 10 pmol of ET-3 to the rostral ET-sensitive area caused a pattern of cardiorespiratory changes identical to the type I response except that the decrease in burst rate was usually absent (Figure 23C). The initial increase of most variables was present but tended to be less conspicuous and blunted. Application of 10 pmol of ET-3 to the caudal ET-sensitive area resulted in a decrease in PNA in all the eight rats examined and an increase in burst rate in all but one animal. Like

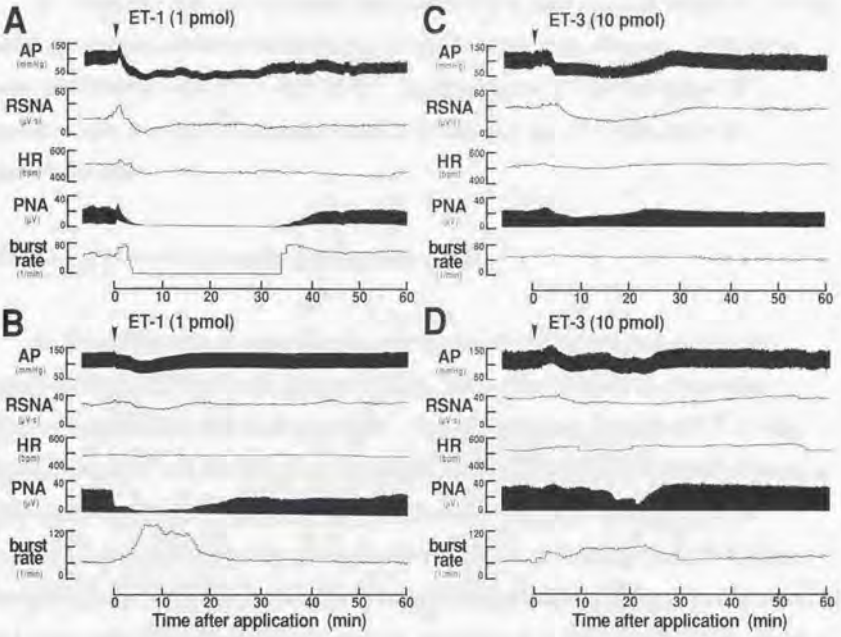


Figure 23 Polygraph records illustrating responses of arterial pressure (AP), renal sympathetic nerve activity (RSNA), heart rate (HR), phrenic nerve activity (PNA), and burst rate of PNA (burst rate) to topical application of 1 pmol of ET-1 (A, B) or 10 pmol of ET-3 (C, D) to the ET-sensitive areas in the VSM. Instantaneously recorded RSNA was integrated over an interval of 10 s and displayed. ET was delivered at the moment marked by arrowheads to a point in the rostral ET-sensitive area in A and C (2.5 mm rostral and 2.0 mm lateral to the reference point) and caudal ET-sensitive area in B and D (0.0 mm rostral and 1.0 mm lateral to the reference point). Each record was obtained from different rats.

the case with ET-1 a moderate decrease in AP with sympathoinhibition, sometimes preceded by a brief increase, accompanied changes in PNA and burst rate (Figure 23D).

In summary, there were rostral and caudal ET-sensitive areas within the VSM which produced characteristic patterns of cardiorespiratory changes. The areas were sensitive to both ET-1 and ET-3. Application of ET to the rostral ET-sensitive area resulted in a similar pattern of changes as with intracisternal administration.

Mapping of rostral and caudal ET-sensitive areas

In the next series of experiments, the extent of the rostral and caudal ET-sensitive areas was explored systematically. Their sensitivity to L-glutamate, glycine and nicotine was also examined . For this purpose, 1 pmol of ET-1 was topically applied and the response magnitude was represented by the peak change in each cardiorespiratory variables. In case the response consisted of an initial transient increase followed by a long-lasting decrease as with the type I response, the latter phase was examined. Since the inhibitory component was much greater and more reproducible than the excitatory component at this dose of ET-1, it more accurately reflected sensitivity to ET.

The rostral ET-sensitive area, in which 1 pmol of ET-1 diminished MAP by more than 40 mmHg and PNA by more than 40% of the control value extended between 2.0 and 3.5 mm rostral and between 1.5 and 2.5 mm lateral to the reference point (Figure 24 A ~ C). The region extended between the caudal border of the trapezoid body and the root of the XIIth nerve rostro-caudally partly overlying the pyramidal tract. It coincided very closely with the glutamate-sensitive area in which 50 nmol of sodium L-glutamate increased MAP by more

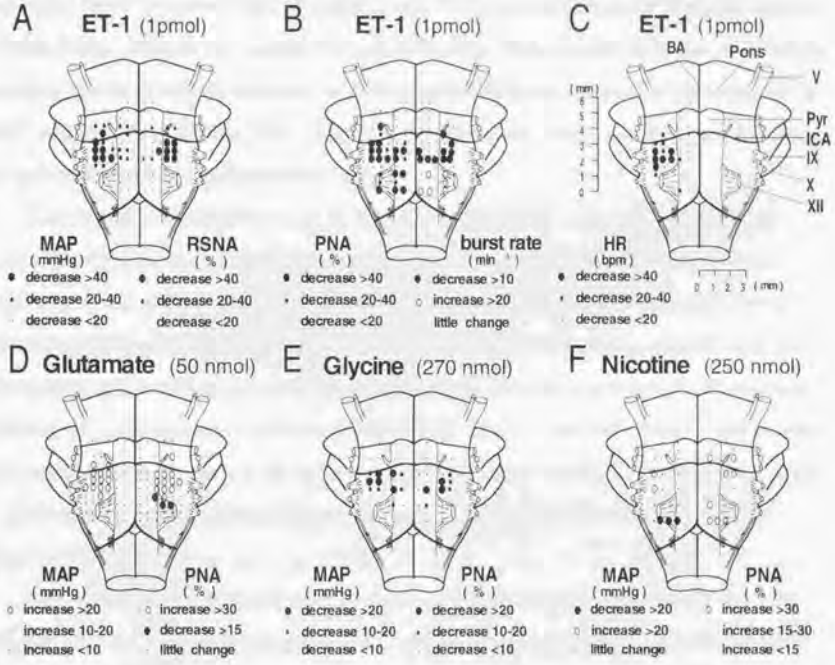


Figure 24 Diagrams of the brain stem of the rat showing points of the ventral surface of the medulla mapped with topical application of ET-1 (A-C), sodium L-glutamate (D), glycine (E), or nicotine (F) for cardiorespiratory responses. The cardiorespiratory variable examined was marked in the right and left halves of each drawing. Dose of the drugs examined was shown in parenthesis. The response magnitude was ranked into 3 levels as noted in each diagram. Distance, in mm, from the reference point, i.e, the caudal end of the basilar artery, is shown in C. Results were obtained from 19 rats for ET-1, 11 for glutamate, 5 for glycine, and 6 for nicotine.

than 20 mmHg and PNA by more than 30% of the control (Figure 24D). It also coincided fairly closely with the glycine-sensitive area where 270 nmol of glycine decreased MAP by more than 20 mmHg and PNA by more than 20% of the control (Figure 24E). Within the rostral ET-sensitive area and around, there were nicotine-sensitive points at which delivery of 250 nmol of nicotine resulted in an increase in MAP and/or PNA (Figure 24F). Such points, however, tended to be scattered and did not constitute a circumscribed region.

The caudal ET-sensitive area in which 1 pmol of ET-1 decreased PNA by more than 40% of control and increased burst rate by more than 20 min^{-1} was located near the rootlet of the XIIth nerve and was much smaller than the rostral counterpart (Figure 24B). The coordinates of this area were between 0.0 and 1.0 mm rostral and between 0.5 and 2.0 mm lateral to the reference point. It was not sensitive to L-glutamate or glycine (Figure 24D and E). In some part of the caudal ET-sensitive area, however, nicotine caused an increase in PNA by more than 30% of control usually accompanied by a decrease in MAP by more than 20 mmHg (Figure 24F).

The effect of ET-3 was then examined by delivering 10 pmol of ET-3 to the central part of the two ET-sensitive areas and the trapezoid body. As described above, ET-3 caused the pattern of cardiorespiratory changes similar to the type I and II responses when applied to the rostral and caudal ET-sensitive areas, respectively. However, no obvious change was brought about when delivered to the trapezoid body.

In summary the rostral ET-sensitive area corresponded to the caudal part of the rostral subdivision and the intermediate subdivision (S area) of the VSM. It also coincided with the glutamate- and glycine sensitive areas. The caudal ET-sensitive area was located near the rootlet of the XII cranial nerve and overlapped with the nicotine sensitive area.

Dose-response relationship of ET-induced cardiorespiratory changes

Dose-response relationship of the peak size of the excitatory and inhibitory components of MAP, RSNA, PNA, burst rate and HR was determined by delivering ET-1 at 5 doses or ET-3 at 3 doses to a point in the central part of the rostral ET-sensitive area (Table 3). In this area topical application of ETs resulted in the pattern of cardiorespiratory changes identical to that following an intracisternal injection (see Section 5-1). Rostro-caudal and lateral coordinates of the point were 2.0 - 3.0 mm and 1.5 - 2.5 mm, respectively.

Application of 0.1 fmol of ET-1 did not cause significant changes. At a dose of 1 fmol, small but significant excitatory components were observed in all the variables except PNA which, however, tended to increase ($P< 0.1$). At a dose of 10 fmol of ET-1, the excitatory component of PNA as well as inhibitory components of MAP, RSNA, and PNA were all statistically significant (Table 3). The inhibitory component of HR was significant at doses of 100 fmol or more and that of burst rate was significant at a dose of 1 pmol. The size of the inhibitory component of all the variables in response to ET-1 was dose-dependent.

In another 21 experiments, ET-3 was given to the same point within the rostral ET-sensitive area. ET-3, at a dose of 1 pmol but not at 0.1 pmol, resulted in significant increases in all the cardiorespiratory variables examined (Table 3). At a dose of 10 pmol but not at 1 pmol, ET-3 caused significant decreases in all the variables except burst rate.

In summary, on application of ET to the rostral ET-sensitive area, the threshold dose for the excitatory component of most of the cardiorespiratory variables examined were below 1 fmol with ET-1 and below 1 pmol with ET-3. That for the inhibitory component was less than 10 fmol with ET-1 and less than 10

TABLE 3. Peak changes in cardiorespiratory variables induced by topical application of ET-1 and ET-3 to the rostral ET-sensitive area in the ventral surface of the rat's medulla

		MAP (mmHg)			RSNA (% of control)		HR (beats/min)			PNA (% of control)		Burst Rate (1/min)		
		Control	Δ MAP		Δ RSNA		Control	Δ HR		Δ PNA		Control	Δ burst rate	
DOSE (fmol)	n		Max of Excitation	Min of Inhibition	Max of Excitation	Min of Inhibition		Max of Excitation	Min of Inhibition	Max of Excitation	Min of Inhibition		Max of Excitation	Min of Inhibition
ET-1														
0.1	6	83±4	2±1	-4±2	8±3	-1±1	513±12	6±2	-2±1	5±3	-5±2	39±5	3±1	-2±1
1	7	81±3	6±2 *	-7±4	22±7 *	-3±3	517±9	7±2 *	-1±1	53±26	-9±5	35±3	9±3 *	-1±1
10	12	91±5	9±2 **	-15±4 **	20±5 **	-23±9 *	504±11	14±3 **	-4±3	32±9 **	-15±4 **	36±2	6±1 **	-2±1
100	9	96±5	5±2 *	-28±5 **	11±3 *	-24±7 **	506±13	6±2 *	-25±9 *	30±6 **	-23±10 *	32±2	11±3 *	-5±4
1000	10	88±4	7±1 **	-35±5 **	24±5 **	-45±7 **	504±11	12±2 **	-40±9 **	35±9 **	-52±12 **	39±2	14±4 **	-22±5 **
ET-3														
100	6	95±6	3±2	-3±1	7±4	-4±3	516±18	4±2	-2±1	1±1	-4±3	35±2	1±1	-3±2
1000	8	98±4	11±3 **	-9±4	38±11*	-4±3	504±14	8±3 *	-3±2	14±6 *	-6±3	36±2	4±1 **	-1±1
10000	7	85±2	5±2 *	-28±4 **	10±4 *	-45±6 **	520±15	9±1 **	-34±13 *	15±5 *	-20±8 *	42±2	7±2 *	-3±2

Values are means ± SE. n = number of experiments. Abbreviations: ET-1, endothelin-1; ET-3, endothelin-3; HR, hart rate; MAP, mean arterial pressure; PNA, phrenic nerve activity; RSNA, renal sympathetic nerve activity. Δ denotes change in each variable from control. Significance of difference from the preinjection control value is denoted: * = P<0.05; ** = P<0.01 (paired t-test).

pmol with ET-3. Thus, ET-1 is more potent than ET-3 by a factor of about 1000, as far as cardiorespiratory responses to ET application to the rostral area are concerned.

6.4 Preliminary Results on the Effect of Intracisternal Administration or Topical Application of Endothelin to the VSM on Medullary Vasomotor Neurons

In a series of preliminary experiments in 12 rats single unit activity of 11 barosensitive neurons were recorded in the RVLM. These neurons were unanimously inhibited by raising arterial pressure by a bolus intravenous injection of phenylephrine (Figure 25B). Five neurons out of 11 (45%) were also antidromically activated by stimulation of descending spinal axons (Figure 25C) and were identified as the vasomotor neurons [Guyenet, 1990]. Antidromic but not orthodromic nature of the activation was confirmed by three criteria [Lipski, 1981]: (1) constant onset latency, (2) high following frequency up to 200 Hz, and (3) the collision test. Spontaneous discharge frequency of the vasomotor neurons was 6.0 ± 1.0 pulses/s at the resting arterial pressure of 87 ± 5 mmHg ($n = 5$). Mean conduction velocity of the descending reticulo-spinal axon of these neurons was 2.0 ± 0.3 m/s.

Three neurons out of 5 vasomotor neurons (60%) responded to topical application of ET-1 (0.1-1 pmol) to the VSM (Figure 25A) or intracisternal injection of 1 pmol of ET-1 in a similar manner as with arterial pressure, namely, initial transient excitation and subsequent inhibition. Onset latency of the excitation was almost equal to that of the pressor response and the entire time course of the response was very similar to that of the biphasic change in arterial pressure. The other two neurons lacked initial excitation but were inhibited by ET during the

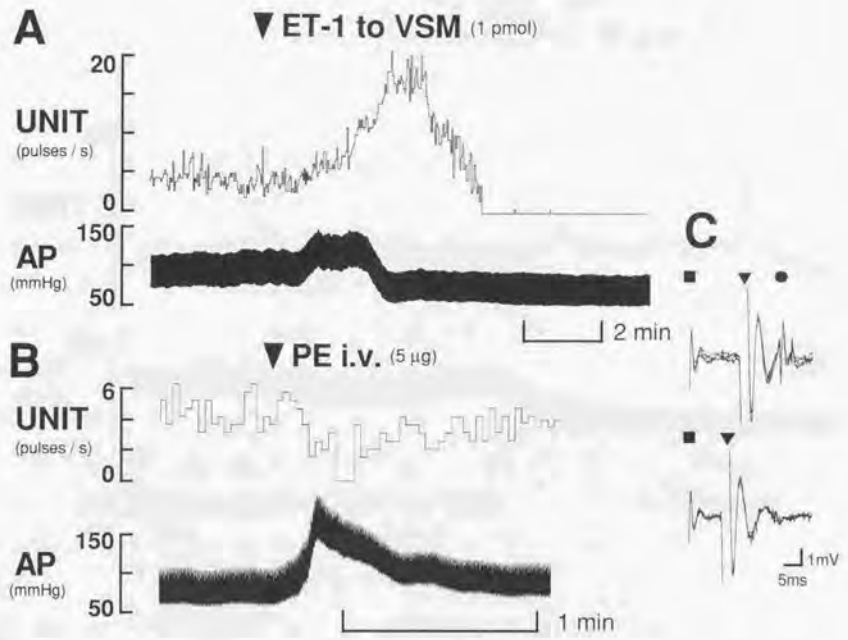


Figure 25 Responses of a barosensitive bulbospinal neuron (UNIT) in the RVLM and arterial pressure (AP) to topically applied ET-1 (1 pmol) to the VSM (A) or intravenous injection of 5 µg of phenylephrine (B). C: Superimposed successive 4 oscilloscope tracings illustrating the collision test for antidromic activation of the barosensitive bulbospinal neuron. stimulating pulses (triangle, 0.5 ms duration, 1 mA intensity) were applied to the spinal sympathetic tract at moments 15 ms (top) and 10 ms (bottom) after spontaneous spikes (rectangle) of the barosensitive bulbospinal neuron. Antidromic spikes (circle) were evoked only after a critical delay.

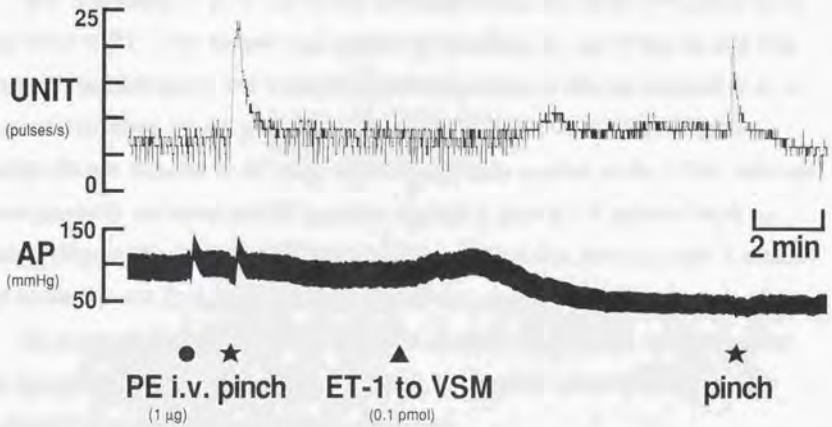


Figure 26 Responses of a nociception-related neuron (UNIT) in the RVLM and arterial pressure (AP) to topically applied ET-1 (0.1 pmol) to the VSM (triangle), nociceptive stimulation to the animal's tail (star, pinching with clumping forceps for 10 s), or intravenous injection of 1 µg of phenylephrine (circle).

depressor period.

Six barosensitive RVLM neurons were not identified as vasomotor, since they were not tested for antidromic activation. However, their response to ET was by and large similar to that of vasomotor neurons. Namely, four of them (67%) showed biphasic change in response to ET, whereas the remaining two neurons showed either initial excitation or subsequent inhibition.

It is interesting to point out in this connection that not all the RVLM neurons responded to ET. One neuron was excited by pinching the tail of the rat and was identified as nociceptive but was not barosensitive since it did not respond to a rise in arterial pressure by i.v. phenylephrine. This nociceptive non-barosensitive neuron did not respond to 0.1 pmol of ET-1 topically applied to the VSM, although simultaneously recorded arterial pressure showed a typical ET-induced biphasic change (Figure 26). Histological examination revealed that this nociceptive neuron was located in the RVLM where 11 barosensitive neurons were distributed.

In summary these preliminary findings strongly suggest that cardiovascular and sympathetic vasomotor changes induced by centrally administered ET are mediated by vasomotor neurons in the RVLM.

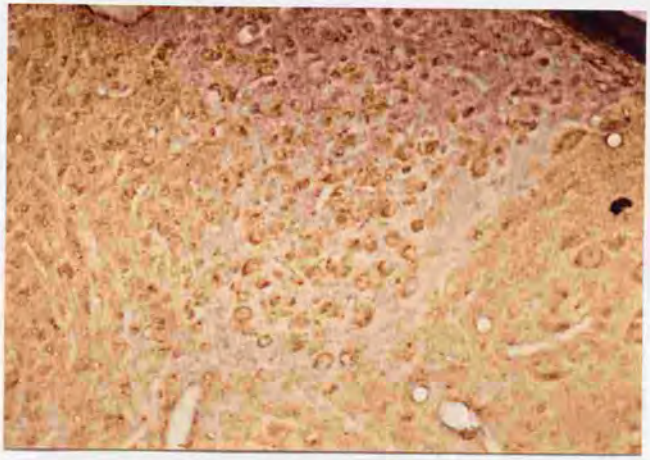
6.5 Immunohistological Study on Distribution of Endothelin-Containing Neurons in the Medulla Oblongata

Some neurons in the NTS, dorsal motor nucleus of vagus nerve (DMN), raphe nuclei, and RVLM were positively stained with either anti-ET-monoclonal antibody or anti-ET antiserum (Figures 27 and 28). However, these positively stained neurones were rather scattered and did not coincide exactly with specific nuclei or neuroanatomical defined regions. Nerve fibers, glial cells, and blood vessels were not visibly immunostained. ET immunoreactivity was abolished following

preabsorption of the antiserum with a mixture of synthetic ET-1 and ET-3 (0.1 nmol/ml diluted antisera). Incubation of sections without one of the steps in the staining procedures did not result in any immunoreaction (Figures 27B and 28B).

In summary ET-containing neurons were identified within certain CNS sites of the medulla oblongata including the RVLM and NTS.

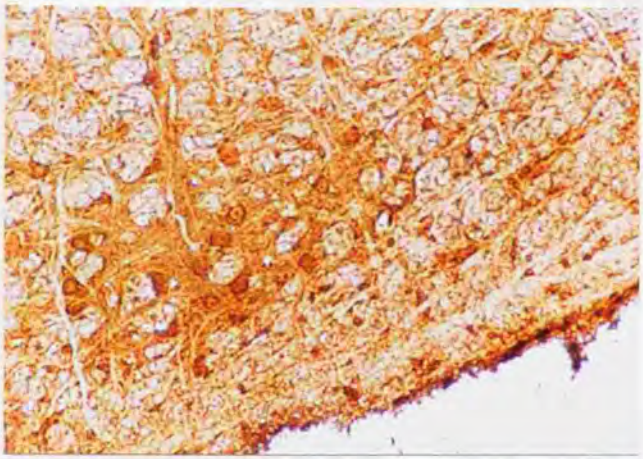
A



B

Figure 27 Endothelin-like immunoreactivity in rat' NTS neurons. A: positive immunostaining. Floor of the 4th ventricle is seen at the right corner of the top side. The frontal section was obtained at 1.0 mm rostral to the calamus scriptorius. Top; dorsal, right; medial. B: negative control showing contralateral side to A. Scale c.a. x 160.

A



B

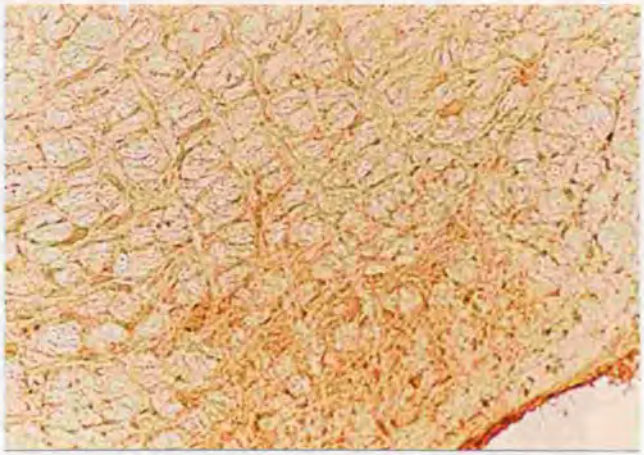


Figure 28 Endothelin-like immunoreactivity in rat' VLM neurons. A: positive immunostaining. Bottom of the medulla oblongata is shown at the right corner of the bottom side. The frontal section was obtained at 2.5 mm rostral to the calamus scriptorius. Top; dorsal, right; lateral. B: negative control. Scale c.a. x 160.

7. DISCUSSION

Effects of intracisternally administered endothelin-1 and endothelin-3 on central cardiorespiratory control

The present study demonstrated that intracisternal administration of ET-1 or ET-3 modulated cardiorespiratory control by altering AP, HR, RSNA, PNA and burst rate. An intracisternal injection of 0.1 pmol of ET-1 or 10 pmol of ET-3 caused a pressor response with sympathoexcitation, tachycardia, and respiratory excitation as evidence by increases in PNA and burst rate of PNA. At a dose of 1 or 10 pmol of ET-1 or 100 pmol of ET-3, a subsequent depressor response with sympathoinhibition, bradycardia, and respiratory inhibition appeared. Especially powerful was the inhibitory effect of ET-1 on respiration. Namely, at a dose as low as 0.1 pmol, ET-1 caused a transient increase followed by longer-lasting decreases in PNA and burst rate. Since the inhibition occurred in vagotomized rats, the observed changes were not secondary to the vagally mediated respiratory reflex but a result of a direct action on the CNS. In line with these observations, Fuxe *et al.* [Fuxe, *et al.*, 1989] reported apnoea in rats anesthetized with α-chloralose on intracisternal administration of 200 pmol of ET-1. In this study, inhibition of respiration occurred at doses much smaller than that. Intracisternal ET-3 also caused an inhibition of PNA. However, the dose required for this effect was 100 pmol. In other words, as far as the modulation of central respiratory control is concerned, ET-1 was more potent than ET-3 by a factor of about 1000. Intracisternally administered ET may cause vasoconstriction of cerebral arteries [Ide, *et al.*, 1989] and ensuing cerebral ischemia might have resulted in the observed cardiorespiratory changes. However, the acute effect of cerebral ischemia is a powerful pressor response with sympathoexcitation and acceleration of respiration

[Dampney, *et al.*, 1979] rather than a complex cardiorespiratory changes observed in the present study. Since a bolus intravenous injection of 100 pmol of ET-3 in the same rat did not cause the pattern of cardiovascular changes comparable to those following intracisternal injection, the observed cardiovascular effect of intracisternally administered ET was centrally mediated but not due to the leakage of the agent to the peripheral circulation.

Although urethane anesthesia is said to distort cardiovascular responses to certain drugs as clonidine [Armstrong, *et al.*, 1982], it is unlikely that the hypotension during phase II in this study was due to this anesthetic agent. Whether the animal was precollicular decerebrated and unanesthetized or anesthetized by urethane, α-chloralose, or pentobarbital, cardiorespiratory responses to ET-1 or ET-3 were very similar. An extraordinarily high dose of ET-1 or ET-3 required to produce comparable cardiovascular and sympathetic changes under pentobarbital anesthesia may be ascribable to its inhibitory effect on the VSM [Feldberg and Guertzenstein, 1976].

Intracisternally administered drug may influence cardiorespiratory control through supramedullary mechanisms such as those mediate by hypothalamic nuclei, e. g., the paraventricular nucleus where vasopressin secreting neurons are located [Day, *et al.*, 1984]. However, pretreatment with systemic administration of vasopressin V_1 antagonist did not seriously distort the cardiovascular or sympathetic response to intracisternally injected ET. Moreover, since precollicular decerebration did not significantly affect the responses to intracisternal ET, the efferent mechanism responsible, either primarily or exclusively, for the effect of intracisternally administered ET on AP and HR was concluded as the sympathetic outflow originated in the brain stem or spinal cord. Interestingly, if ET-1 is given to the lateral or third ventricle of the rat, the dose required to elicit a pressor response comparable to those of phase I in the present study was in the order of 10

to 100 pmol [Minamisawa, *et al.*, 1989; Ouchi, *et al.*, 1989] as against 0.1 to 10 pmol for intracisternal administration in this study. This result suggests that the effective site of cardiovascular and sympathetic vasomotor actions of ET-1 in the cerebrospinal fluid, either endogenously or exogenously supplied, is in the lower brainstem rather than the forebrain.

Although ET-1 and ET-3 are both members of the same ET family, distinct response to the each peptide may be elicited, since two subtypes of receptors for ET have been identified and found in the brain [Arai, *et al.*, 1990; Sakurai, *et al.*, 1990]. The ET_A receptor has high specificity to ET-1 [Arai, *et al.*, 1990], whereas ET_B receptor is non-selective and accepts the three subtypes of ET almost equally [Sakurai, *et al.*, 1990]. Distribution of binding sites and tissue contents within various portions of the rat's brain differ considerably between ET-1 and ET-3 [Fuxe, *et al.*, 1989; Matsumoto, *et al.*, 1989]. Nevertheless, as demonstrated in this study, intracisternal administration of ET-1 and ET-3 elicited a very similar pattern of changes in AP, RSNA, HR, PNA, and burst rate of PNA. ET-1, however, was much more potent in eliciting these changes than ET-3. Namely, the dose of ETs that caused a pressor response with sympathoexcitation, tachycardia and respiratory acceleration was 0.1 pmol with ET-1 and 10 pmol with ET-3. On the other hand, 1 or 10 pmol of ET-1 and 100 pmol of ET-3 both elicited changes in AP, RSNA, HR, PNA and burst rate characterized by phases I and II. Moreover, a threshold dose to elicit respiratory inhibition was less than 0.1 pmol with ET-1 and 100 pmol with ET-3. As discussed below, similarity of the response pattern and difference in the potency appears to be primarily attributable to the responsiveness of the VSM to the two peptides.

In addition to modulation of tonic control of cardiovascular and respiratory activity by the CNS, intracisternally administered 1 or 10 pmol of ET-1 and 100 pmol of ET-3 modulated the reflex control as evidenced by suppression of the

arterial baroreceptor reflex, peripheral and central chemoreceptor reflexes, and somato-sympathetic reflex. When peripheral chemoreceptor reflex was inhibited by intracisternal ET, inhalation of N_2 elicited a depressor response rather than the pressor response seen in the control period. A similar depressor response was also observed in surgical deafferentation of arterial chemoreceptors and thought to be of peripheral origin that had been overridden by reflex sympathoexcitation when chemoreceptors were intact [Sun and Reis, 1992]. This observation supports the view that the observed inhibition of the peripheral chemoreceptor reflex by ET was not secondary to the depressor effect of ET.

Brain sites responsible for modulatory action of centrally administered endothelin on cardiorespiratory control

Precollicular decerebration did not seriously altered cardiovascular and respiratory changes induced by ET. Furthermore, intracisternally administered ET-1 and ET-3 both affected tonic sympathetic and phrenic nerve discharges as well as the arterial baroreceptor reflex, peripheral and central chemoreceptor reflexes, and somato-sympathetic reflex. However, the effect of intrathecal injection of these peptides on AP, RSNA, and HR was minute, if any. Consequently, it was expected that the major effective CNS site of intracisternally administered ET met the following criteria. (i) It was within the midbrain or ponto-medullary region. (ii) It participated in generation or regulation of tonic sympathetic discharges. (iii) It was located along the central pathway subserving or modulating the arterial baroreceptor reflex, peripheral and central chemoreceptor reflexes, and somato-sympathetic reflex. The VSM, a restricted area in the ventrolateral surface of the medulla oblongata [Bruce and Cherniack, 1987; Trouth, *et al.*, 1973] which is functionally and neuroanatomically associated very closely to the RVLM

[Benarroch, *et al.*, 1986; Sun, *et al.*, 1991], a key CNS site with respect to maintenance of tonic sympathetic vasomotor discharges [Ross, *et al.*, 1984], meets all these criteria. Namely, mechanically or pharmacologically induced impairment of the VSM results in a marked decrease in AP with sympathoinhibition [Guertzenstein and Silver, 1974; Wennergren and Oberg, 1980] and impairs the arterial baroreceptor-sympathetic vasomotor reflex [Saeki, *et al.*, 1988]. The VSM has been regarded as the site of central chemoreceptors which is responsive to a variety of chemical agents in addition to CO_2 and H^+ [Bruce and Cherniack, 1987].

As expected, topical application of 1 pmol of ET-1 or 40 pmol of ET-3 to the VSM resulted in the pattern of changes almost identical to those following intracisternal injection, although the actual amount of ET released from the filter paper topically applied to the VSM and acted on the VSM was unknown. On the other hand, a local microinjection of ET-1 or ET-3 into the intermediate region of the NTS, usually covering its medial and ventrolateral subnuclei [Kalia, *et al.*, 1984], resulted in long lasting increases in AP, RSNA, HR, PNA, and burst rate, pattern of which was completely different from that with intracisternal or VSM application of ET. Futhermore, when ET was applied to the area postrema either by direct injection or through the vertebral artery, cardiorespiratory responses were small inhibition or a no significant changes, respectively. Collectively, these findings support the view that the VSM is involved critically in the effect of intracisternally administered ET-1 or ET-3 on the central cardiorespiratory control.

The results of topical application of ET to the NTS is in line with abundant presence of binding sites for ET-1 in the rat's NTS [Koseki, *et al.*, 1989; Koseki, *et al.*, 1989]. Since the effective dose range for ET-1 and ET-3 was similar (more than 0.1 pmol), the major type of receptors in the NTS involved in the observed cardiorespiratory changes appears to be ET_B receptors, which have similar affinity

to ET-1 and ET-3 [Sakurai, *et al.*, 1990]. Furthermore, since ET-1 and ET-3 caused similar cardiorespiratory changes at comparable doses, the observed cardiorespiratory responses were a consequence of the direct effect on neural elements but not a result secondary to vasoconstriction. In fact, vasoconstriction by ET in the peripheral circulation is attributed to ET_A receptors distributed in the vascular smooth muscle cells [Sakurai, *et al.*, 1992].

Although ET-1 and ET-3 elicited cardiorespiratory changes over a similar dose range, there were significant differences in the magnitude of cardiorespiratory changes (Table 2 and Figure 19). Namely, ET-1 elicited preferentially respiratory changes, whereas ET-3 particularly elicited cardiovascular changes at certain doses. This observation raises a possibility that there may be putative ET_C receptors specific to ET-3 as suggested in the pituitary [Samson, *et al.*, 1990]. Additional experiments using selective antagonists are needed to elucidate the precise reason for the difference in the effect of ET-1 and ET-3.

In line with the present observation Ferguson and Smith [Ferguson and Smith, 1990] reported a depressor response in urethane-anesthetized rats on microinjection of ET-1 to the area postrema at a dose of 2 pmol or more. They also found that intravenously injected ET-1 (2 pmol) influenced the single unit activity of the spontaneous active neurons in the area postrema (excitatory; 45%, inhibitory; 8%, unaffected; 47% in 60 neurons tested), although AP did not change [Ferguson and Smith, 1991]. Although the area postrema is accessible to ET in the peripheral circulating blood, the amount of ET to affect neuronal activity is too high as compared to normal plasma level of ET (normally 0.2 - 1.6 fmol/ml in human plasma [Fujimori, *et al.*, 1990; Kaufman, *et al.*, 1991; Schiffrin and Thibault, 1991; Yamaji, *et al.*, 1990]).

In the excised spinal cord of the newborn rat, ET-1 added to the perfusion medium caused depolarization of the ventral root potential in a dose-dependent

manner [Yoshizawa, *et al.*, 1989]. Presence of ET-1 mRNA in certain regions of the spinal cord including the intermediolateral nucleus was reported by a study employing in situ hybridization technique [Giaid, *et al.*, 1989]. Therefore, it is puzzling that intrathecally administered ET-1 and ET-3 affected only moderately at a dose as high as 100 pmol in the present study. Two possible explanations, not mutually exclusive though, are (i) neonatal rats are more sensitive to ET than adult counterparts, and (ii) intrathecally administered ET in our experiments did not permeate deep enough to reach the intermediolateral nucleus.

Subregion of the ventral surface of the medulla responsible for central cardiorespiratory effect of endothelin

In the present study two separate ET-sensitive areas were identified within the rat's VSM in which ET elicited distinct patterns of cardiorespiratory changes termed the type I and II responses. Of these, the type I response was identical to that following an intracisternal injection of ET. It was produced by application of ET to the rostral ET-sensitive area which was also sensitive to L-glutamate.

The glutamate-sensitive area in the rat's VSM is responsive to a variety of substances including kainic acid, carbachol and γ-aminobutyric acid [Benarroch, *et al.*, 1986] and overlaps with the RVLM which participates in tonic and reflex control of sympathetic vasomotor discharges [Ciriello, *et al.*, 1989]. Furthermore, the reticulospinal sympathetic premotor neurons with intrinsic pacemaker activity in the RVLM have dendritic projections reaching a subregion of the VSM [Sun, *et al.*, 1991] which corresponds to the rostral ET-sensitive area. It is suggested that cardiovascular changes elicited from the VSM is mediated by neurons of the RVLM [Benarroch, *et al.*, 1986]. Electrical stimulation of this area or application of carbachol to it in rats increased the depth and frequency of respiration [Malcolm, *et*

al., 1980]. Conceivably, the rostral ET-sensitive area of the VSM and subjacent RVLM not only mediates cardiorespiratory responses to topically or intracisternally administered ET but also serves as an integral part in the central cardiorespiratory control. Findings in the present study that ET delivered to the glutamate-sensitive area of the VSM affected both circulation and respiration and that vasomotor neurons in the RVLM responded to ET applied either intracisternally or locally to the VSM strongly support this hypothesis.

The precise site of action of ET, or distribution of the putative ET receptor in the rostral ET sensitive area in a cytological level, e.g., at dendrite or at soma, is not yet known. Superficially applied drugs are reported to penetrate into the brain parenchyma. Gamma-aminobutyric acid which has molecular weight of 103 penetrates by 500 μm within 2 min when applied to the VSM [Keeler, *et al.*, 1984]. Since ET have molecular weight of 2492 (ET-1) or 2643 (ET-3) and the diffusion coefficient of globular molecules in water is roughly inversely proportional to the square or cube root of the molecular weight [Martin, 1964], it can be estimated that ET may penetrate into parenchyma about 200 to 300 μm in 2 min. According to the report by Macrae *et al.* [Macrae, *et al.*, 1991], [^{125}I]ET-1 invaded over a distance of less than 50 μm in 2 min. When ET was applied to the rostral ET-sensitive area in the VSM, the onset latency of cardiorespiratory changes was less than one minute. This time span is inadequate for ET to reach the soma of vasomotor neuron in the RVLM which are located between 200 and 1,000 μm deep from the ventral surface [Sun, *et al.*, 1991]. At least during the initial period of the response to topically applied ET to the VSM, ET seems to act on dendrites of the vasomotor neuron, which run as closely as 20 to 30 μm from the surface of the brain [Sun, *et al.*, 1991].

There was a separate region near the rootlet of the XIIth cranial nerve in which ET caused the type II response characterized typically by an inhibition of

PNA with an increase in burst rate. In certain part of the caudal ET-sensitive area, nicotine (250 nmol) caused an increase in PNA with a decrease in AP. The area, which did not elicit significant cardiorespiratory changes on application of L-glutamate, was located more caudal to the depressor points observed by McAllen in the cat's VSM [McAllen, 1986]. Anyhow, it is interesting to point out that there is a subregion of the rat's VSM in which ET-1 affected respiration with less effects on circulation.

Another new finding of the present study is that ET-1 delivered to the rostral ET-sensitive area was more potent than ET-3 by a factor of about 1000. Although ET-1 is much more potent than ET-3 in many instances, the opposite is also true with respect to certain effects of ET. For example, when injected intravenously into anesthetized rats, ET-3 causes a greater initial transient depressor response than ET-1, whereas ET-3 is much weaker than ET-1 as a pressor agent [Inoue, *et al.*, 1989]. As to the CNS effect, ET-3 produces a greater increase in RSNA than ET-1 when microinjected into the NTS of the rat (see above). The finding on the high potency of ET-1 may indicate that the cardiorespiratory response to ET, whether intracisternally given or locally delivered to the rostral ET-sensitive area, is mediated primarily by ET_A receptor, which has a higher specificity to ET-1 than to ET-3 [Arai, *et al.*, 1990].

The cardiorespiratory effect of topically applied ET may be ascribable to the change in the microenvironment surrounding neurons such as ischemia as a result of cerebral vasoconstriction. The acute effect of cerebral ischemia on circulation, however, is a powerful pressor response with sympathoexcitation [Dampney, *et al.*, 1979] rather than the pattern of cardiorespiratory changes termed type I and II responses in this study. The long-lasting action of ET may suggest participation of a hormonal or paracrine mechanism. ET-1 most likely stimulates formation of nitric oxide in a cultured neuronal cell line (mouse neuroblastoma x rat glioma

hybrid cells) [Reiser, 1990]. Moreover, locally released nitric oxide is said to affect signal transmission, e.g., in the cerebellum [Garthwaite, 1991]. Involvement of such a mechanism in the central cardiorespiratory effect of ET may not be impossible but highly speculative. Rather, considering a long-lasting effect of ET on some target cells such as the vascular smooth muscle [Yanagisawa, *et al.*, 1988] it seems more plausible that the cardiorespiratory responses to topically applied ET is a consequence of its direct action on neural elements in the VSM and perhaps the RVLM.

Effects of intracisternal administration or topical application of ET to the VSM on medullary vasomotor neurons

The rostral ET-sensitive area in the VSM is highly sensitive to ET to elicit cardiorespiratory modulation. I, therefore, tried to test the hypothesis whether the vasomotor neuron in the RVLM may be involved in the cardiovascular response to topical application of ET to the VSM. Since the rostral ET-sensitive area coincided with the glycine-sensitive area which constituted the ventral surface of the RVLM [Benarroch, *et al.*, 1986; Guyenet, 1990], this hypothesis seemed promising. Moreover, dendritic processes of the vasomotor neuron in the RVLM extend very close to the VSM [Sun, *et al.*, 1991]. The vasopressor effect of L-glutamate applied to the VSM is mediated by the RVLM because the effect was abolished by lesioning the RVLM [Benarroch, *et al.*, 1986].

In the preliminary study using single-unit recording technique, activity of five vasomotor neurons in the RVLM was recorded. Their electrophysiological characteristics were consistent with the reported property of vasomotor neurons in the rat's RVLM [Brown and Guyenet, 1985; Chan, *et al.*, 1991; Morrison, *et al.*, 1988] with respect to their spontaneous discharge rate, cardiac-related fluctuations

of the spontaneous discharge, and the conduction velocity of descending spinal axons. As expected, on delivery of ET-1 to the VSM three of the 5 vasomotor neurons tested showed a biphasic change with an initial excitation and subsequent inhibition. The remaining two neurons lacked the initial excitatory phase. On the other hand, a nociception-related non-cardiovascular neuron in the RVLM did not respond to ET-1, although the arterial pressure showed a typical biphasic change. These results, although preliminary, strongly support the hypothesis that vasomotor neurons in the RVLM is involved, at least in part, in the cardiovascular effect of application of ET to the VSM. To my knowledge this is the first report which shows that application of a chemical agent to the VSM causes a change in activity of vasomotor neurons in the RVLM.

The lack of responsiveness of the nociception-related neuron in the RVLM to ET-1 suggests that the change in activity of vasomotor neurons is not due to vasoconstriction induced by ET-1 and consequent hypoxia. Ischemia-induced hypoxia should have indiscriminately affected vasomotor and non-vasomotor neurons alike. In this connection Sun *et al.* [Sun, *et al.*, 1992] have recently reported that the vasomotor neuron in the RVLM was excited by iontophoretically applied cyanide while respiratory and other neurons of unidentified modality were inhibited. They proposed that vasomotor neurons were endowed with chemosensitivity to hypoxia/ischemia. Therefore, difference in sensitivity to hypoxia rather than that to ET between vasomotor and nociception-related neurons may explain the observed results. However, the threshold dose of microinjected ET-1 to induce a decrease in regional blood flow in the cerebral cortex [Willette and Sauermelch, 1990] was greater than that of topical application to the VSM to induce cardiorespiratory response in the present experiments (10 fmol vs. 1 fmol). Notwithstanding, the precise mechanism of excitation and inhibition of vasomotor neuron in response to topically applied ET to VSM is still unresolved.

Immunohistological study on distribution of ET-containing neurons in the medulla oblongata

Some neurons in the NTS, DMN, raphe nuclei, and RVLM showed ET-like immunoreactivity. This ET-like immunoreactivity was attributable to ET-1 and/or ET-3, since both anti-ET-monoclonal antibody and anti-ET antiserum used in the present experiment have almost equal affinity to ET-1 and ET-3 [Saito, *et al.*, 1990]. In this connection Giaid *et al.* [Giaid, *et al.*, 1991] have reported presence of the ET-like immunoreactivity and ET mRNA in neurons of DMN and raphe nuclei of the human brain. They also reported that the number of cells which expressed mRNA was much greater than that of neurons containing the mature peptide of that mRNA. They interpreted the result to mean that the concentration of the peptides diminished rapidly due to the lack of storage or rapid release after death. This possibility may explain demonstration of ET-like immunoreactivity in neurons of the NTS and VLM in addition to the DMN and raphe nuclei in the present experiments in which the rat's brains was rapidly fixed. Failure to identify the ET-like immunoreactivity in the nerve fibers or in glial cells leaves a possibility of a role of ET as a local neuropeptide. Through this type of experiment, it is impossible to determine whether those immuno-positive neurons are cardiorespiratory-related or not. However, both ET and ET-receptors can express themselves in the same hypothalamic neuron in culture [Krsmanovic, *et al.*, 1991]. Together with the concept of "ET as the local hormone acting in an autocrine or paracrine fashion" [MacCumber, *et al.*, 1990], such observation raises a possibility that the ET-containing neurons in the NTS or in the VLM may be cardiorespiratory-related and have ET-receptors. Combining the present results with the fact that the NTS, DMN, and VLM have ET-binding sites (putative receptors) [Gulati and

Rebello, 1991; Koseki, *et al.*, 1989; Koseki, *et al.*, 1989], it is strongly suggested that ET plays some role as a neuropeptide or a neuromodulator within local neuronal networks involved in central cardiorespiratory control.

General discussion and future studies

In the present study, it is shown that centrally administered ET-1 or ET-3 affected tonic and reflex control of the circulation and respiration by the CNS. Most effective site in the lower brainstem was a discrete region in the VSM termed the rostral ET-sensitive area. Moreover, vasomotor neurons in the RVLM, which control primarily the sympathetic outflow to blood vessels and hence regulate AP, was shown to mediate the cardiovascular effect of topically applied ET to the VSM.

Since ET is a powerful vasoconstrictor, observed cardiorespiratory responses to exogenously applied ET in this study might be a secondary effect of vasoconstriction of cerebral arteries and ensuing cerebral ischemia. However, following lines of evidence suggest that ET affects directly on the neurons. First, acute effect of cerebral ischemia on circulation is a powerful pressor response with sympathoexcitation [Dampney, *et al.*, 1979] rather than the pattern of cardiorespiratory changes termed type I and II responses in this study. Second, the threshold dose of microinjected ET-1 to induce a decrease in regional blood flow in the cerebral cortex [Willette and Sauermelch, 1990] was greater than that of topical application to the VSM to induce cardiorespiratory response in the present experiments (10 fmol vs. 1 fmol). Third, ET affected spontaneous activities of the hypothalamic neurons even in the slice preparations [Yamashita, *et al.*, 1991], in which oxygen was supplied to the neurons through perfusion fluid.

Although the minimum dose of ET-1 to the rostral ET-sensitive area required for cardiorespiratory effects was as small as 1 fmol, the present study does not tell

us whether the area actually participates in the central cardiorespiratory control under physiological or pathophysiological settings. However, ET is present in the human CSF at concentrations around 0.1 to 33 pM [Ando, *et al.*, 1991; Fujimori, *et al.*, 1990; Kraus, *et al.*, 1991; Shirakami, *et al.*, 1990; Suzuki, *et al.*, 1990; Suzuki, *et al.*, 1990; Togashi, *et al.*, 1990; Yamaji, *et al.*, 1990]. Some investigators have claimed that during the course of subarachnoid hemorrhage its concentration increases parallel to the severity of the cerebral vasospasm [Kraus, *et al.*, 1991; Shirakami, *et al.*, 1990; Suzuki, *et al.*, 1990; Suzuki, *et al.*, 1990], although no change was reported in other studies [Fujimori, *et al.*, 1990; Yamaji, *et al.*, 1990]. Furthermore, neurons in certain CNS sites such as the paraventricular nucleus of the hypothalamus or DMN of the medulla oblongata contain mRNA of ET and are positive in the ET-like immunoreactivity [Giaid, *et al.*, 1991; Lee, *et al.*, 1990; Yoshizawa, *et al.*, 1990]. The present study showed that some neurons in NTS and RVLM also have ET-like immunoreactivity. The rat's medulla oblongata contains relatively high amount of ET-1 and ET-3 [Matsumoto, *et al.*, 1989] and the concentration of ET-1 in the medulla is lower in developing spontaneously hypertensive rats than normotensive Wistar Kyoto rats [Yoshimi, *et al.*, 1991]. Moreover, ET receptors are confirmed in the rat's VLM [Gulati and Rebello, 1991].

These pieces of evidence collectively support the view that ET circulating in the CSF is monitored by neurons in the rostral ET-sensitive area of the VSM and/or vasomotor neurons in the RVLM and affects tonic and reflex control of the circulation and respiration. Futhermore, ET may serve as a neurotransmitter or neuromodulator in the RVLM and other neural sites that participate in the central cardiorespiratory control at least under certain situations.

Since specific ET-receptor antagonists have been developed recently [Ihara, *et al.*, 1991; Sogabe, *et al.*, 1992], it is now possible to test whether endogenously

present but not exogenously applied ET in the CSF or in vasomotor neurons of the RVLM contribute to control of tonic activity of vasomotor neurons and basal activity of the phrenic nerve as well as reflex control of the cardiorespiratory system.

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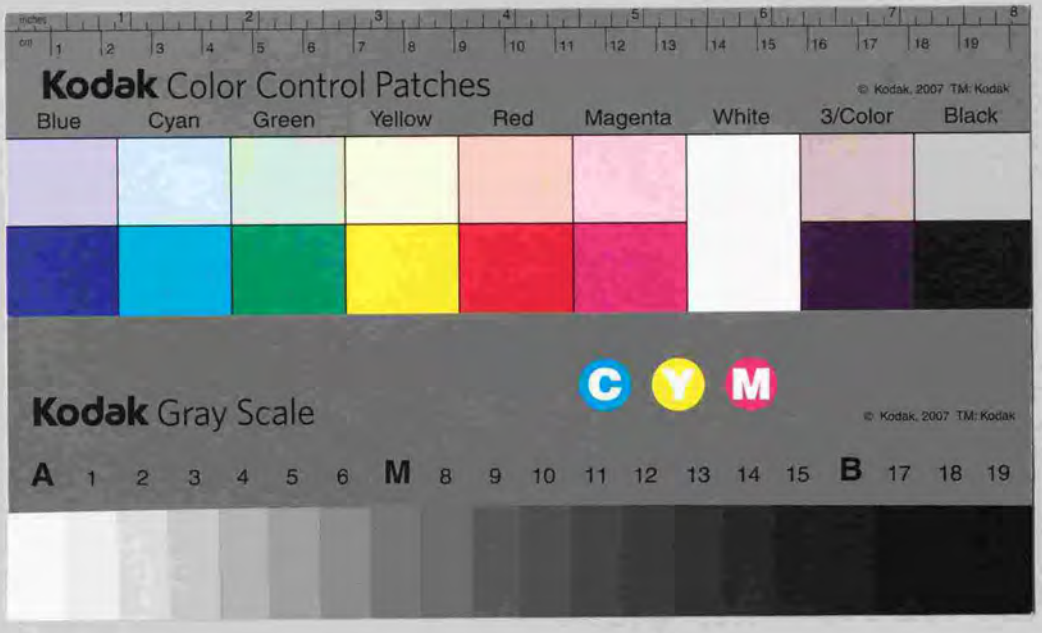
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