Endogenous Calcitonin Gene-Related Peptide Mediates Nonadrenergic Noncholinergic Depressor Response to Spinal Cord Stimulation in the Pithed Rat

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The role of endogenous calcitonin gene-related peptide (CGRP) in the nonadrenergic noncholinergic depressor response to spinal cord stimulation was studied in the pithed rat in vivo. Pithed rats were given hexamethonium (2 mg/kg per minute i.v.) to block autonomic outflow, and mean blood pressure was artificially maintained at approximately 100 mm Hg with methoxamine (10–15 μg/kg per minute i.v.). Electrical stimulation of the spinal cord at the level of the lower thoracic vertebrae (T9-12) caused a fall in blood pressure in a frequency-dependent (0.5–10 Hz), voltage-dependent (2.5–50 V), and pulse duration-dependent (0.25–8 msec) manner. The heart rate did not change during the depressor response. The depressor response was long lasting, and the maximum response was elicited by stimulation at 4–6 Hz. The neurotoxin tetrodotoxin (100 μg/kg i.v.) abolished the depressor response to spinal cord stimulation, whereas treatment with propranolol (0.5 mg/kg per minute i.v.), atropine (0.05 mg/kg per minute i.v.), or a combination of pyrilamine (0.5 mg/kg per minute i.v.) and cimetidine (0.5 mg/kg per minute i.v.) did not affect the response. In pithed rats treated with capsaicin (total dose of 500 mg/kg s.c.), spinal cord stimulation caused a slight depressor response. Exogenous CGRP, but not acetylcholine, isoproterenol, histamine, or substance P, caused a sustained fall in blood pressure that mimicked the spinal cord stimulation-induced depressor response. Continuous infusion of CGRP[8-37] (60 nmol/kg per minute i.v.), a CGRP receptor antagonist, markedly inhibited the depressor responses not only to spinal cord stimulation but also to exogenous CGRP. These results suggest that spinal cord stimulation causes nonadrenergic noncholinergic vasodilation and that the response is mediated by endogenous CGRP, which is probably released from capsaicin-sensitive and CGRP-containing nerve terminals.

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Key Words: calcitonin gene-related peptide • nonadrenergic noncholinergic depressor response • CGRP-containing nerve terminals

The tone of peripheral resistance blood vessels is mainly regulated by sympathetic adrenergic nerves through release of the neurotransmitter norepinephrine. However, nonadrenergic nerves innervate regional vascular beds, and nonadrenergic vasodilation is observed in various species.1 The exact nature of the neurotransmitter substance of nonadrenergic nerves is not fully determined. Recent in vitro studies have shown that perivascular nerve stimulation of the perfused rat mesenteric vascular bed causes neurogenic vasodilation, which is mediated by nonadrenergic noncholinergic (NANC) nerves.2 The peptidergic and sensory neurotoxin capsaicin, which depletes calcitonin gene-related peptide (CGRP) and substance P from perivascular nerves,4–6 abolishes NANC vasodilation.2,3 In addition, perivascular nerve stimulation causes the tetrodotoxin-sensitive release of CGRP associated with the vasodilation.7,8 Therefore, we proposed that CGRP is a potential neurotransmitter for NANC vasodilation of mesenteric resistance blood vessels.2 More recent studies with a specific CGRP receptor antagonist (CGRP[8-37]), CGRP receptor desensitization, and antisera against CGRP have confirmed that the NANC vasodilation in the rat mesenteric vascular bed is mediated by endogenous CGRP, probably released from CGRP-containing vasodilator nerve terminals.5,10 CGRP, a 37 amino acid peptide translated from the calcitonin gene,11 is a potent vasodilator12,13 and is widely distributed in perivascular nerves throughout the vascular system.14,15 The distribution and vascular activity of CGRP may indicate that it is an important endogenous neurotransmitter for neural regulation of the cardiovascular system. However, the role of CGRP-containing nerves in cardiovascular regulation in vivo remains unknown. Therefore, the present study was designed to investigate 1) whether NANC vasodilation in vivo is elicited by spinal cord stimulation in the rat and 2) the possibility that CGRP is an endogenous
neurotransmitter that mediates NANC vasodilation. To achieve these aims, we used pithed rats with artificially increased blood pressure (BP) and with the autonomic outflow blocked.

**Materials and Methods**

**Animals**

Male Wistar rats, weighing 360–420 g, were used in this study. The animals were given food and water ad libitum. They were housed in the experimental animal center of Miyazaki Medical College at a controlled ambient temperature of 22°C with 50±10% relative humidity and with a 12-hour light–dark cycle (light on at 7:30 AM).

**Pithing and Measurements**

The animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Polyethylene catheters (PE-10) were positioned in the right and left jugular veins for administration of drugs, and a bilateral vagotomy was performed at the midcervical level. A catheter (PE-50) was inserted into the left carotid artery and was connected to a Statham pressure transducer (model P23ID, Gould, Cleveland, Ohio). The arterial BP was recorded on a polygraph (model RM-6000, Nihon Kohden, Tokyo). The heart rate (HR) triggered by arterial pulses was measured by a cardiotachometer (model AT-600G, Nihon Kohden) and was recorded on the polygraph.

After the trachea was cannulated, the animals were pithed by inserting a stainless-steel rod (1.5 mm in diameter) through the right orbit and the foramen magnum and down into the spinal cord to the level of sacral end, and then the tip of the rod was raised to the thoracolumbar vertebra, according to the method described by Gillespie.16,17 Artificial respiration (4.5 ml/beat per kilogram, 70 beats per minute) with room air was immediately started using an artificial respirator (model 680D, Harvard Apparatus, South Natick, Mass.). The pithing rod served as the stimulating electrode, which was insulated except for 5 mm of the tip. The level of spinal cord stimulation was determined by varying the depth of insertion of the rod. The position of the rod within the vertebral canal was investigated by radiography in some rats and was determined from the length of the rod. A stainless-steel needle was inserted under the skin of the back, parallel to the vertebral column, to serve as an indifferent electrode. After the animals were pithed, d-tubocurarine (1 mg/kg i.v.) was injected to prevent skeletal muscle contraction during spinal cord stimulation. The rectal temperature was maintained at 37°C with a heating pad.

**Spinal Cord Stimulation**

After allowing BP and HR to stabilize, electrical stimulation (rectangular pulses at 10 V, 1-msec pulse duration) was applied to verify the position of the rod in the spinal column. Then, mean BP was increased and maintained at a level of approximately 100 mm Hg by continuous infusion of the q1-adrenergic agonist methoxamine (10–15 μg/kg per minute i.v.). Hexamethonium (2 mg/kg per minute i.v.) was also infused to block autonomic outflow. The increased BP was allowed to stabilize, and then the spinal cord was again electrically stimulated: Rectangular pulses were given for 30 seconds by varying frequencies (0.5, 1, 2, 4, 6, and 8 Hz, at 10 V and 1-msec duration), voltages (2.5, 5, 7.5, 10, 25, and 50 V, at 4 Hz and 1-msec duration), and pulse durations (0.25, 0.5, 1, 2, 4, and 8 msec, at 4 Hz and 10 V) with an electronic stimulator (SEN 3301, Nihon Kohden).

**Capsaicin Treatment**

The animals were anesthetized with halothane and were treated with 1 mg/kg i.p. atropine, and then they were injected subcutaneously with 100 mg/kg capsaicin or an equal volume of solvent (50% ethanol in 0.9% saline) on the first day; the capsaicin was given in four injections (5, 15, 30, and 50 mg/kg) at 3-hour intervals. On the two following consecutive days they were treated with doses of 200 mg/kg per day capsaicin (four injections of 50 mg/kg s.c. at 3-hour intervals), giving a total dosage of 500 mg/kg, or with an equal volume of solvent, according to the method described by Jessel et al.18 Five days after the last injection, the animals were subjected to pithing.

**Statistical Analysis**

The experimental results are expressed as mean±SEM. Statistical significance was determined by one-way analysis of variance followed by Dunnett’s test. A value of p<0.05 was considered statistically significant.

**Drugs**

The following drugs were used: acetylcholine chloride (Daichi Pharmaceutical Co., Tokyo), atropine sulfate (Sigma Chemical Co., St. Louis, Mo.), capsaicin (Sigma), cimetidine (Sigma), guanethidine sulfate (Tokyo Kasei, Tokyo), hexamethonium bromide (Sigma), histamine dihydrochloride (Wako Junyaku, Osaka, Japan), human CGRP[8-37] (Peptide Institute, Osaka, Japan), t-iso-proterenol hydrochloride (Sigma), propranolol hydrochloride (Sigma), pyrilamine maleate (Sigma), rat CGRP (Peptide Institute), substance P (Peptide Institute), and tetrodotoxin (Sigma). Capsaicin was dissolved in 50% ethanol in 0.9% saline and injected subcutaneously. All other drugs were dissolved in 0.9% saline and infused at a rate of 0.3 ml/hr using an infusion pump (model 11, Harvard Apparatus) or were given as bolus doses (0.2 ml/kg).

**Results**

**Cardiovascular Responses to Spinal Cord Stimulation**

Electrical stimulation of the higher thoracic region (T1-4) evoked a sharp rise in BP and an increase in HR (Figure 1A). Stimulation (2–8 Hz) of the lower thoracic region (T9-12) evoked a frequency-dependent increase in BP without alteration of HR (Figure 1B). Therefore, in the following experiments the lower thoracic region (T9-12) was stimulated to minimize the cardiac effect on vascular responses. As shown in Figure 1B, pressor responses to spinal cord stimulation were completely inhibited by the autonomic ganglionic blocker hexamethonium (2 mg/kg per minute i.v.). The pressor responses were also abolished by the neurotoxin tetrodotoxin (100 μg/kg i.v.) and by the adrenergic neuron blocker guanethidine (5 mg/kg per minute i.v.) (data not shown). In some cases, spinal cord stimulation
caused a small fall in BP after autonomic outflow was blocked with hexamethonium, as shown in Figure 1C.

**Depressor Response to Spinal Cord Stimulation**

The mean BP of pithed rats was artificially increased and maintained at approximately 100 mm Hg by continuous infusion of methoxamine (10–15 μg/kg per minute i.v.) in the presence of hexamethonium (2 mg/kg per minute i.v.). As shown in Figure 1C, electrical stimulation (10 V, 1-msec duration) of the lower thoracic region (T9-12) produced a frequency-dependent fall in BP. This depressor response appeared 10–20 seconds after the stimulation began and reached a maximum at 1–2 minutes after the stimulation ended, and BP returned to the prestimulation level within 5–15 minutes in the case of higher frequencies (4–8 Hz). The HR did not change during the depressor response, and there was no tachyphylaxis.

Figure 2 shows that the depressor response to spinal cord stimulation was dependent on voltage, frequency, and pulse duration of the stimulus. Higher voltages (25–50 V) and pulse durations (2–8 msec) caused a twitching of the skeletal muscle despite the presence of d-tubocurarine. Also, the depressor response induced by 10 Hz was significantly smaller (p<0.01) than responses induced by 4 and by 6 Hz. Thus, the optimum frequency, voltage, and pulse duration of stimulation were 4–6 Hz, 10 V, and 1 msec, respectively.

**Effects of Various Drugs on the Pressor Response to Spinal Cord Stimulation**

As shown in Figures 1D and 3, the frequency-dependent depressor response to spinal cord stimulation was abolished by tetrodotoxin (100 μg/kg i.v.). However, the depressor response to spinal cord stimulation (4 Hz) was not affected by treatment with the β-adrenoceptor antagonist propranolol (0.5 mg/kg per minute i.v.) or with the muscarinic cholinergic antagonist atropine (0.05 mg/kg per minute i.v.) at a dose that effectively antagonized the depressor response to a bolus injection of the β-adrenoceptor agonist isoproterenol (1 nmol/kg i.v.) or of the cholinergic agonist acetylcholine (1 nmol/kg i.v.) (Figures 4A, 4B, and 5). Combined infusion of pyrilamine (0.5 mg/kg per minute i.v.), a histamine H₂-receptor antagonist, and cimetidine (0.5 mg/kg per minute i.v.), a histamine H₃-receptor antagonist, had no effect on the depressor response to spinal cord stimulation (4 Hz), whereas both antagonists abolished the depressor response to a bolus injection of histamine (0.3 nmol/kg i.v.) (Figures 4C and 5).
Depressor Responses to Bolas Injections of Various Vasodilators

Figure 6 shows the depressor responses to various vasodilators and to spinal cord stimulation in the pithed rat with artificially increased BP. As shown in Figure 6A, spinal cord stimulation caused a frequency-dependent and long-lasting fall in BP without changes in HR. Bolus injection of rat CGRP (0.1 nmol/kg i.v.) also caused a long-lasting fall in BP without changes in HR, which mimicked the depressor response to spinal cord stimulation. However, bolus injections of acetylcholine (1 nmol/kg i.v.), substance P (0.3 nmol/kg i.v.), isoproterenol (1 nmol/kg i.v.), and histamine (0.3 nmol/kg i.v.) induced a sharp and transient fall in BP (Figures 6B and 6C). Injection of isoproterenol, but not acetylcholine, substance P, or histamine, increased HR.

Effect of Capsaicin Treatment

As shown in Figure 7, spinal cord stimulation in vehicle-treated pithed rats caused a frequency-dependent depressor response. In pithed rats treated with capsaicin (total doses of 500 mg/kg s.c.), depressor responses to spinal cord stimulation were significantly smaller than in vehicle-treated pithed rats.

Effect of CGRP[8-37], a CGRP Receptor Antagonist

As shown in Figure 8A, both spinal cord stimulation and bolus injection of rat CGRP caused a long-lasting fall in BP without changes in HR, whereas bolus injection of isoproterenol induced a transient fall in BP concomitant with an increase in HR. Continuous infusion of human CGRP[8-37] (60 nmol/kg per minute i.v.) resulted in a slight increase in BP (14±2 mm Hg, n=7) and markedly inhibited depressor responses to exogenous rat CGRP and to spinal cord stimulation (Figure 8B). After cessation of human CGRP[8-37] infusion, the depressor responses to spinal cord stimulation and to rat CGRP returned to the levels measured before infusion of the antagonist (Figure 8C). However, neither the depressor response nor the increase in HR caused by isoproterenol was affected by human CGRP[8-37] (Figure 8B). Figure 9 summarizes the changes in depressor responses induced by spinal cord stimulation (2 and 4 Hz), exogenous rat CGRP, and isoproterenol before, during, and after infusion of human CGRP[8-37].

Discussion

The pithed rat preparation has been used to study cardiovascular changes mediated by autonomic outflow from the spinal cord in vivo. Previous reports and the present study show that electrical stimulation of the thoracolumbar spinal cord in the pithed rat increases the pressor response with little change in HR. Since this response can be attenuated by an autonomic ganglionic blocker (hexamethonium), an adrenergic neuron blocker (guanethidine), and an α-adrenoceptor antagonist (phenolamine), the response results from vasoconstriction mediated by norepinephrine that is released in response to preganglionic stimulation of
sympathetic efferent nerves. Thus, the pithead preparation is useful for studying precise mechanisms of central factors involving blood pressure control. However, stimulation of the spinal cord causes both ventral and dorsal root activation. Therefore, it appears that hemodynamic changes induced by spinal cord stimulation in the presence of hexamethonium result from activation of fibers other than autonomic nerves.

In the present experiment, to observe the depressor response in pithead rats, mean BP was artificially increased and maintained at a level of 100 mm Hg by continuous infusion of methoxamine (a selective α₁-adrenoceptor agonist) in the presence of hexamethonium. Since infusion of methoxamine did not alter the baseline HR, it is likely that the elevated BP resulted from vasoconstriction induced by stimulation of vascular α₁-adrenoceptors. In the pithead rat with artificially increased BP, spinal cord stimulation produced a frequency-dependent fall in BP, even in the presence of hexamethonium. However, HR did not change during the fall in BP. Therefore, it is very likely that this depressor response was mainly due to the reduced peripheral resistance that resulted from vasodilation.

The neurotoxin tetrodotoxin abolishes the depressor response to spinal cord stimulation, suggesting that the response is neurogenic. Blood vessels in the skin and skeletal muscles of the limbs are supplied by several populations of sympathetic vasodilator neurons. The vasodilator responses mediated by these neurons are atropine and hexamethonium sensitive, indicating that sympathetic cholinergic nerves are involved. However, this is not the case in the present results, because hexamethonium was always infused to block autonomic outflow and because the depressor response to spinal cord stimulation was not sensitive to propranolol or atropine at the dose that abolishes the depressor response to isoproterenol and to acetylcholine. These results strongly suggest that NANC nerves are responsible for the depressor response to spinal cord stimulation. Endogenous histamine has also been proposed to be involved in the neurogenic vasodilation of the skin and hind leg, because the vasodilation is inhibited by the histamine receptor antagonist, especially by histamine H₁-receptor antagonist, and is also sensitive to the autonomic ganglionic blocker. However, the present findings show that the depressor response to spinal cord stimulation is not sensitive to the histamine H₁- and H₂-receptor antagonist. Thus, involvement of endogenous histamine in the NANC depressor response to spinal cord stimulation is unlikely. Furthermore, the present study shows that the depressor response to spinal cord stimulation is long lasting and that this differs from the responses to a β-adrenoceptor agonist (isoproterenol), to a cholinergic agonist (acetylcholine), and to histamine, in which the responses are quite transient. Taken together, these results clearly indicate that adrenergic, cholinergic, and histaminergic mechanisms do not participate in the spinal cord stimulation-induced depressor response.

Capsaicin markedly inhibited depressor responses to spinal cord stimulation. This suggests that the neurogenic depressor response is capsaicin sensitive and is mediated by substances released from capsaicin-sensitive sensory neurons. At least 12 different types of
peptidyl immunoreactive substances, including CGRP, substance P, somatostatin, and vasoactive intestinal polypeptide, have been reported to be present in capsaicin-sensitive sensory neurons.23 Among them, CGRP-containing fibers are widely distributed in the adventitious wall of blood vessels.15,24 In addition, perivascular nerve stimulation of isolated mesenteric vascular beds produces increased release of CGRP and vasodilation, both of which are sensitive to tetrodotoxin and capsaicin.7,8 Therefore, it is very likely that endogenous CGRP is responsible for the NANC depressor response to spinal cord stimulation. On the other hand, CGRP has been shown to coexist with substance P,7,24 and both CGRP and substance P are potent vasodilators.11,25 The present study shows that exogenous CGRP causes a sustained depressor response that mimics the spinal cord stimulation–induced depressor response, whereas substance P produces a transient depressor response similar to that caused by acetylcholine. Furthermore, substance P and acetylcholine, but not CGRP, are very potent endothelium-dependent vasodilators.26 The long distance between the nerve terminals and endothelial cells makes it unlikely that substance P released from nerves reaches the endothelial cells in sufficient concentrations to induce vasodilation. Thus, it

**FIGURE 7.** Graph showing the effect of pretreatment with capsaicin (500 mg/kg s.c.) and vehicle (5 ml/kg s.c.) on the depressor responses to spinal cord stimulation in pithed rats. The ordinate indicates percent decrease in mean blood pressure (MBP), which is expressed as percentages of MBP increased by continuous infusion of methoxamine (10–15 μg/kg per minute i.v.). The abscissa shows frequency of the stimulus. Data are shown as mean±SEM. *p<0.01 and **p<0.001 compared with vehicle control.

**FIGURE 8.** Typical recordings of the effect of human calcitonin gene-related peptide [8-37] (CGRP[8-37]) on the depressor responses to spinal cord stimulation (2 and 4 Hz) and to bolus injections (circles) of rat calcitonin gene-related peptide (CGRP, 0.1 nmol/kg i.v.) and isoproterenol (ISO, 1 nmol/kg i.v.) in the pithed rat. BP, blood pressure; HR, heart rate; C6, hexamethonium; ISO, isoproterenol; MBP, mean BP. BP was artificially increased by methoxamine in the presence of C6. Panel A: Control responses. Panels B and C: Responses during and after continuous infusion of CGRP[8-37], respectively.

**FIGURE 9.** Bar graph showing the effect of human calcitonin gene-related peptide [8-37] (CGRP[8-37]) on the depressor responses to spinal cord stimulation (2 and 4 Hz) and to bolus injection of rat calcitonin gene-related peptide (CGRP, 0.1 nmol/kg) and isoproterenol (1 nmol/kg) in pithed rats. The ordinate indicates percent decrease in mean blood pressure (MBP), which is expressed as percentages of MBP increased by continuous infusion of methoxamine (10–15 μg/kg per minute i.v.). The open, closed, and dotted columns (mean±SEM) indicate the responses before, during, and after infusion of CGRP[8-37], respectively. *p<0.01 and **p<0.001 compared with values before or after infusion of CGRP[8-37] as indicated by the thin lines.
is unlikely that substance P is involved in the depressor response to spinal cord stimulation.

Recently, the C-terminal fragment of human CGRP, human CGRP[8-37], has been demonstrated to dose-dependently replace 

\[ ^{125}\text{I-}[\text{Y}^\text{r}]	ext{rat CGRP binding and to inhibit adenylate cyclase activation induced by CGRP in rat liver plasma membranes, suggesting that human CGRP[8-37] is a CGRP receptor antagonist.} \]

Human CGRP[8-37] abolishes the positive inotropic response of the isolated guinea pig left atrium to exogenous CGRP, electrical field stimulation, or capsaicin.\(^{28}\) It also inhibits both the vasodilation caused by exogenous CGRP and by perivascular nerve stimulation in the perfused mesenteric vascular bed.\(^{9}\) An in vivo study using conscious and pentobarbital-anesthetized rats also showed that human CGRP[8-37] antagonizes the hypotensive response to exogenous CGRP.\(^{29,30}\) In addition, the selectivity of human CGRP[8-37] has been confirmed by the finding that it has no effect on the vasodilator response to bradykinin, histamine, isoproterenol, or substance P.\(^{9,30}\) In the present study, human CGRP[8-37] markedly inhibited the depressor responses to spinal cord stimulation and to exogenous rat CGRP but not to isoproterenol. This finding strongly suggests that the NANC depressor response is mediated by endogenous CGRP, which is probably released from capsaicin-sensitive and CGRP-containing nerve terminals by spinal cord stimulation.

Direct electrical stimulation of the dorsal spinal column and of the peripheral part of cut dorsal roots or sensory nerves (saphenous nerves or trigeminal nerves) has been shown to result in vasodilation in the skin and skeletal muscle area supplied by these nerves.\(^{31-34}\) It is well known that this vasodilation is produced by antidromic conduction in sensory nerves. The depressor response to spinal cord stimulation is sensitive to capsaicin, and CGRP is contained in capsaicin-sensitive sensory nerves. Therefore, the spinal cord stimulation-induced depressor response may result from antidromic conduction of sensory afferent nerves. This conduction may cause the release of CGRP from distal terminals of sensory nerves, which bifurcate from the sensory nerves.\(^{35}\) However, many studies have shown that antidromic stimulation does not change the systemic blood pressure and that the regional vasodilation produced is sensitive to atropine, histamine antagonists, and indomethacin.\(^{31-34}\) Such results are not consistent with the present findings that stimulation of a limited spinal segment (T9-12) can cause a systemic depressor response that is insensitive to atropine and to histamine antagonists. Therefore, we hypothesize that there is outflow of CGRP-containing vasodilator nerves from the spinal cord to blood vessels. Further experiments are needed to test this hypothesis.

In conclusion, the present study demonstrates that spinal cord stimulation results in a neurogenic depressor response, which is mediated by NANC vasodilator nerves. Endogenous CGRP is the most likely mediator of this vasodilation and is probably released from capsaicin-sensitive nerve terminals by electrical stimulation of the spinal cord. The present report is the first to provide in vivo evidence that endogenous CGRP acts as a neurotransmitter that mediates NANC vasodilation. The present results also suggest that CGRP-containing vasodilator nerves play a role in neuronal control of the tone of resistance blood vessels.

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