Decreased Depressor Response Mediated by Calcitonin Gene-Related Peptide (CGRP)-Containing Vasodilator Nerves to Spinal Cord Stimulation and Levels of CGRP mRNA of the Dorsal Root Ganglia in Spontaneously Hypertensive Rats

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The depressor response to electrical stimulation of the spinal cord and the level of calcitonin gene-related peptide (CGRP) mRNA in the dorsal root ganglion (DRG) in the spontaneously hypertensive rat (SHR) was compared with the normotensive Wistar Kyoto rat (WKY) and Wistar rat (WR). The animals were pithed by inserting a stainless-steel rod into the spinal cord. Pithed rats were treated with hexamethonium (2 mg/kg/min i.v.) to block autonomic outflow, and mean arterial blood pressure (MBP) was maintained at approximately 100 mmHg with continuous infusion of methoxamine (10 to 15 µg/kg/min i.v.). Electrical stimulation (2 and 4 Hz for 30 s) of the lower thoracic spinal cord (T9-12) via the pithing rod caused a frequency-dependent depressor response without a change in heart rate. The depressor response to spinal cord stimulation was significantly smaller in SHR than in WKY and WR. Long-term treatment of 8-week-old SHR with captopril (0.1% in drinking water) for 7 weeks restored the reduced depressor response to spinal cord stimulation. The level of CGRP mRNA in DRG of SHR was significantly lower than that in WKY. These results suggest that the function of CGRP-containing nerves from the spinal cord decreases in SHR and captopril treatment prevents its reduction. (Hypertens Res 2000; 23: 693-699)

Key Words: calcitonin gene-related peptide (CGRP) vasodilator nerve, spinal cord stimulation, depressor response, pithed rat, SHR, CGRP mRNA

Introduction

The resistance artery is innervated by non-adrenergic non-cholinergic (NANC) vasodilator nerves, which play a role in the regulation of vascular tone (1, 2). We have demonstrated that the rat mesenteric resistance artery is innervated by NANC vasodilator nerves in which calcitonin gene-related peptide (CGRP), a potent vasodilator neuropeptide, acts as the vasodilator neurotransmitter (1, 3, 4). CGRP is distributed throughout the central and peripheral nervous systems and is located in areas involved in car-
diovascular function (5). A prominent site of CGRP synthesis is the dorsal root ganglia (DRG), which contains the cell bodies of primary afferent neurons that extend CGRP-containing nerves (CGRPergic nerves) to peripheral sites such as blood vessels and the central spinal cord (6). CGRPergic nerves suppress vasoconstrictor responses to adrenergic nerve stimulation through released CGRP, and conversely, adrenergic nerves inhibit the release of CGRP from the nerve to decrease CGRPergic nerve function (3, 4). Thus, we have proposed that CGRPergic vasodilator nerves along with sympathetic vasoconstrictor nerves regulate the tone of the mesenteric resistance artery. Furthermore, *in vivo* studies on pithed rats showed that electrical stimulation of the spinal cord at the lower thoracic spinal cord (T9-12) induces a NANC nerve-mediated neurogenic depressor response without a change in heart rate (HR) (7, 8). This response is inhibited by CGRP (8-37), a CGRP receptor antagonist, suggesting that CGRPergic nerves mediate the response (7, 8).

The increased total peripheral vascular resistance maintains elevated blood pressure in chronic hypertension (9, 10) and the impaired function of the control systems regulating peripheral resistance has been considered (11, 12). In studies with spontaneously hypertensive rats (SHR), the enhanced activity of sympathetic vasoconstrictor nerves contributed to the increased tone of peripheral resistance arteries (11-13). We have demonstrated that the CGRPergic nerve function in SHR decreases with age, and proposed that malfunction of CGRPergic vasodilator nerves regulating peripheral vascular resistance plays an important role in the development and maintenance of hypertension in SHR (14, 15). However, the mechanisms underlying the reduced function of CGRPergic vasodilator nerves in SHR remain unresolved. In recent pharmacological studies, we showed that angiotensin (Ang) II inhibits neurotransmission of CGRPergic nerves in SHR (16) and chronic treatment of SHR with the Ang-converting enzyme (ACE) inhibitor but not other antihypertensive drugs such as a calcium antagonist and β-adrenoceptor antagonist prevents the decrease in vasodilator responses mediated by CGRPergic nerves (17, 18).

The present study was, therefore, undertaken to investigate the outflow of CGRPergic nerves from the spinal cord and the levels of CGRP mRNA of DRG in SHR. Baroreflex function in SHR has been shown to decrease with age (19). To avoid intervention of baroreflex, the present experiment was done in pithed animals.

**Methods**

**Animal**

Male SHR at 8 or 15 weeks of ages and normotensive Wistar Kyoto rats (WKY) and Wistar rats (WR) at 15 weeks of age (purchased from Charles River Japan, Shizuoka, Japan) were used for this study. The animals were given food and water *ad libitum*. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C with 50±10% relative humidity and with a 12-h light/12-h dark cycle (light on at 8:00 AM).

**Pithing and Measurement**

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 polyethylene catheter (PE-50) was inserted into the left carotid artery and was connected to a pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan), and blood pressure (BP) was recorded on a polygraph (RM-6000, Nihon Kohden). The heart rate (HR) triggered by arterial pulses was measured by a cardiotachometer (AT-600G, Nihon Kohden).

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 (T9-12), according to the method described previously (7, 8, 20). Artificial respiration (4.5 ml/beat/kg, 70 beats/min) with room air was immediately started using an artificial respirator (model 680D, Harvard Apparatus, South Natick, USA). 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 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-ms pulse duration) at 4 Hz, which induced an increase in BP without changing HR, was applied to verify the position of the rod in the spinal cord. Then, mean BP (MBP) was increased and maintained at a level of approximately 100 mmHg by continuous infusion of the α1-adrenoceptor agonist methoxamine (10 to 15 µg/kg/min i.v.). The autonomic ganglionic blocker, hexamethonium (2 mg/kg/min i.v.), was also infused to block the autonomic outflow. The increased BP was allowed to stabilize, and then the spinal
cord was again electrically stimulated: Rectangular pulses (1 ms in duration and 10 V) at 2 and 4 Hz were given for 30 s with an electronic stimulator (SEN 3310, Nihon Kohden).

Measurement of CGRP mRNA

Fifteen week-old SHR and age-matched WKY were anesthetized with pentobarbital-Na (50 mg/kg i.p.) and the left carotid artery was cannulated to measure BP using a pressure transducer. At the end of the experiment, the animals were deeply anesthetized with a large dose of pentobarbital-Na and killed by decapitation. The thoracic and lumbar dorsal root ganglia (DRG), kidney, atria and thyroid gland from each rat were immediately dissected and frozen in liquid nitrogen until subsequent analysis of CGRP mRNA.

Total cellular RNA was prepared using the AGCP method (the acid guanidium thyocyanate-phenol-chloroform method) (21). The quality of extracted RNA samples was confirmed by measurement of absorbance of 260 nm in a spectrophotometer. The RNA samples were fractionated by electrophoresis on denaturing formaldehyde-agarose gels and transferred to a nylon membrane. Hybridization was done under stringent condition (50% formamide at 42°C) with a 32P-labelled cDNA probe. Three probes for CGRP, β-actin and G3PDH (glyceraldehyde-3-phosphate dehydrogenase) were labeled, using a Multi-prime DNA labeling system (Amersham Int. plc., Buckinghamshire, UK) with 32P-dCTP. After hybridization, the membranes were washed and exposed to X-ray films. The radioactivity of each band was quantified by means of computerized densitometric scanning (BAS 2000, Fuji Photo Film Co., Tokyo, Japan). All results are expressed relative to the β-actin mRNA, serving as a control for the quality and quantity of RNA applied to the blot.

Long-Term Treatment with Captopril

Male SHR at 8 weeks of age received 0.1% captopril (100 mg/kg/day) in their drinking water and normal rat chow for a period of 7 weeks. Non-treated control SHR received normal drinking water and rat chow. The treated SHR at 15 weeks of age was anesthetized and the pithing was done as described above.

Statistical Analysis

Data are presented as the mean±SEM. One-way analysis of variance followed by Tukey's test was used to determine the significance between values of different ages or different doses. Unpaired Student's t test was used to determine the significance of differences between two means. A value of p<0.05 was considered significant.

<table>
<thead>
<tr>
<th>Animals</th>
<th>MBP before pithing (mmHg)</th>
<th>MBP after pithing (mmHg)</th>
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<tbody>
<tr>
<td>SHR (6)</td>
<td>187.7±1.5</td>
<td>44.7±2.2</td>
</tr>
<tr>
<td>WKY (4)</td>
<td>97.0±1.8**</td>
<td>38.8±1.7</td>
</tr>
<tr>
<td>WR (7)</td>
<td>95.5±2.7**</td>
<td>39.4±1.2</td>
</tr>
<tr>
<td>Capt-SHR (4)</td>
<td>95.0±2.9**</td>
<td>38.3±4.4</td>
</tr>
</tbody>
</table>

Values show mean±SEM. **p<0.01, compared with SHR. (n) number of animals.

Drugs

The following drugs were used: hexamethonium bromide (Sigma Chemical Co., St. Louis, USA), methoxamine hydrochloride (Nihon Shinyaku, Kyoto, Japan), captopril (Sankyo Pharmaceutica Co., Tokyo, Japan), human CGRP (8-37) (Peptide Institute, Osaka, Japan) and rat α-CGRP (Peptide Institute). All drugs were dissolved in 0.9% saline and infused at a rate of 0.3 ml/h using an infusion pump (model 11, Harvard Apparatus, South Antic, USA) or were given as a bolus dose (0.2 ml/kg i.v.).

Results

MBP in Pithed SHR, WKY and WR

As shown in Table 1, the MBP before pithing was significantly greater in SHR than in WKY or WR. There was no significant difference in the MBP after pithing between SHR and WKY or WR.

Depressor Responses to Spinal Cord Stimulation

In the pithed rat with an arterial BP increased by continuous infusion of methoxamine (10 to 15 μg/kg/min i.v.) in the presence of hexamethonium (2 mg/kg/min i.v.), spinal cord stimulation (2 and 4 Hz) induced a frequency-dependent decrease in arterial BP, as shown in Fig. 1. The depressor response appeared 10 to 20 s after stimulation began and reached a maximum 1 to 2 min after the stimulation ended. No change in HR was observed during the depressor response (Fig. 1). The depressor response to spinal cord stimulation was inhibited by CGRP (8-37) at a dose of 60 nmol/kg/min i.v. (data not shown). We have reported that the depressor response to spinal cord stimulation was abolished by the neurotoxin, tetrodotoxin (100 μg/kg i.v.) and inhibited by infusion of CGRP (8-37) (7, 8).

As shown in Figs. 1 and 2A, spinal cord stimulation (2 and 4 Hz) in both WR and WKY induced a frequency-
dependent depressor response without affecting HR. However, in SHR, spinal cord stimulation (2 and 4 Hz) caused a small depressor response without changes in HR. There were significant differences in depressor responses to spinal cord stimulation between SHR and normotensive controls (WKY and WR) (Fig. 2A).

Long-term treatment with captopril for 7 weeks significantly lowered arterial BP of SHR before the pithing, compared with non-treated control SHR (Table 1). In the captopril treated SHR, depressor responses to spinal cord stimulation (2 and 4 Hz) were significantly greater than those in non-treated SHR and similar to the response in WKY and WR, as shown in Fig. 2B.

CGRP mRNA Level

In this series of experiments, MBP of SHR (190.8±2.6 mmHg, n=5) at 15 weeks of age was significantly greater (p<0.01) than age-matched WKY (92.2±2.9 mmHg, n=5)

Figure 3A shows a representative Northern blot analysis of CGRP mRNA. Content of CGRP mRNA in DRG of WKY and SHR was rich. Little or no level of CGRP mRNA was found in the atria, kidney or thyroid gland (data not shown). When the level of CGRP mRNA in SHR was compared with WKY as the ratio of CGRP mRNA/β-actin mRNA, a significant decrease was found in SHR, as shown in Fig. 3B.

**Discussion**

The present findings confirmed previous studies that electrical stimulation (2 and 4 Hz) of the lower thoracic spinal cord (T9-12) in the pithed rat produced a frequency-dependent fall in arterial BP which was artificially increased by continuous infusion of methoxamine, an α₁-adrenoceptor agonist. This depressor response was mainly due to reduced peripheral vascular resistance that resulted from vasodilation because HR did not alter during the response. In previous pharmacological studies, the depressor response to spinal cord stimulation was not inhibited by atropine (muscarinic cholinoceptor antagonist), propranolol (β-adrenoceptor antagonist), pyrilamine (histamine H₁ receptor antagonist), or abolished by the neurotoxin, TTX, or by the CGRP receptor antagonist, CGRP (8-37) (7). This indicates that the response is NANC in nature and mediated by endogenous CGRP released from the CGRPergic nerves (7).

*In vitro* studies on the rat mesenteric artery and *in vivo* studies on the pithed rat showed that capsaicin, a sensory afferent neurotoxin, inhibits the vasodilation mediated by CGRPergic nerves (1, 7). Moreover, we have previously shown that acute and chronic multiple dorsal root rhizotomy of the rat abolishes the depressor response to spinal cord stimulation (8). Therefore, it is possible that the antidromic conduction of sensory afferent nerves is responsible for the vasodilation induced by spinal cord stimulation. However, the depressor response is induced by
stimulation of a limited segment of the spinal cord (T9-12) but not (T1-4) (7). Antidromic stimulation has been shown to cause no change in systemic BP (22). Thus, we proposed that CGRPergic vasodilator nerves may course from the spinal cord to peripheral blood vessels via the dorsal roots (7, 8).

In in vitro studies, we have reported that NANC vasodilation in response to periarterial nerve stimulation (PNS) of the mesenteric artery in SHR is smaller than WKY and the decrease in the response is age-related (14). However, vasodilator response to exogenous CGRP in the mesenteric artery of SHR is greater than WKY, suggesting that vascular CGRP receptors in SHR may be up-regulated due to decreased function of CGRPergic nerves (14). Since the PNS-induced NANC vasodilation is mediated by CGRPergic nerves, the decreased vasodilator response to PNS indicates the reduced function of CGRPergic nerves in SHR. The present study demonstrated that the depressor response to spinal cord stimulation in SHR is much smaller than in normotensive WR and WKY. This finding strongly suggests that the outflow of CGRPergic nerve activity decreases in SHR.

The amount of CGRP mRNA in the DRG in 12-week-old SHR has been shown to decrease when compared with age-matched WKY (23). In addition, the content of CGRP in the dorsal horn of the spinal cord has been reported to be lower in 12-14 week old SHR than WKY (24). In the present study, 15-week-old SHR had significantly decreased levels of CGRP mRNA compared with age-matched WKY. Taken together, these results support the present finding in the in vivo study that the outflow of CGRPergic nerve decreased in SHR. CGRPergic nerves have been shown to inhibit the function of adrenergic nerves (3, 25, 26). Therefore, the decreased function of CGRPergic nerves in SHR enhances the sympathetic adrenergic vasoconstriction. A recent study demonstrated that α-CGRP/calcitonin gene knockout mice displayed an elevated MBP compared with wild-type mice, indicating that α-CGRP is involved in the long-term regulation of resting BP (27). Therefore, the reduced CGRPergic nerve function may cause the increased vascular resistance and high blood pressure.

In in vitro studies, we have shown that the reduced vasodilation mediated by CGRPergic nerves in SHR mesenteric arteries is restored by long-treatment with ACE inhibitor, captopril and temocapril (17, 18). However, ACE inhibitors did not affect the vasodilator response to exogenous CGRP (18). Neither the vasodilator (hydralazine), β-adrenoceptor antagonist (propranolol) nor calcium antagonist (nicardipine) have such an effect despite lowering MBP. Furthermore, Ang II as well as Ang I and renin substrate (N-acetyltetradecapeptide) has been shown to inhibit the neurotransmission of CGRPergic nerves in SHR but not WKY (16). In the present in vivo study, long-term treatment of SHR with the ACE inhibitor, captopril, lowered the blood pressure and restored the reduced depressor response induced by spinal cord stimulation to a level similar to normotensive rats. Thus, the ACE inhibitor has an ability to improve the decreased CGRPergic nerve function in SHR. Therefore, we proposed that circulatory Ang II as well as the converted one in the vasculature is responsible for the decreased function of CGRPergic nerves in SHR. Deng et al. (28) reported the increased level of Ang II in the heart of 15
week-old SHR than age-matched WKY, suggesting that the Ang II level may increase in DRG of SHR. This may cause the reduced CGRPergic function in DRG.

In conclusion, the present study indicates that the CGRPergic nerve-mediated depressor response to stimulation of the lower thoracic spinal cord and CGRP gene expression in DRG decrease in SHR. The present study suggests that the function of CGRPergic vasodilator nerves from the spinal cord decreases in SHR and this decrease contributes to maintain hypertension in SHR.

References

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