Effects of Long-Term Treatment With Calcium Antagonists on Periarterial Nerve Function in the Mesenteric Artery of Spontaneously Hypertensive Rats

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ABSTRACT—The effect of long-term treatment with dihydropyridine calcium antagonists (amlodipine, pranidipine, nicardipine) on the periarterial nerve function was investigated in the perfused mesenteric vascular bed isolated from spontaneously hypertensive rat (SHR). Male 8-week-old SHR received amlodipine (0.01% and 0.02%) and nicardipine (0.1%) in drinking water and pranidipine (0.0035% and 0.035%) in rat chow for 7 weeks. Mean blood pressure in SHR was significantly lowered by long-term treatment with each calcium antagonist. In mesenteric vascular preparations treated with each calcium antagonist, vasoconstriction induced by periarterial nerve stimulation (PNS; 4, 8 and 12 Hz) was significantly smaller than that in non-treated SHR. The PNS (8 Hz)-evoked norepinephrine (NE) overflow in the perfusate was significantly decreased by amlodipine and pranidipine treatment, whereas nicardipine-treatment significantly enhanced the overflow of NE. In preparations with active tone produced by methoxamine and guanethidine, the PNS-induced vasodilation mediated by calcitonin gene-related peptide (CGRP)-containing (CGRPergic) vasodilator nerves was not affected by these drugs. These results suggest that long-term treatment of SHR with long-acting drugs, amlodipine and pranidipine, reduces sympathetic adrenergic nerve function but calcium antagonists have no effect on CGRPergic nerve function.

Keywords: Calcium antagonist, Adrenergic vasoconstrictor nerve, Calcium gene-related peptide-containing vasodilator nerve, Spontaneously hypertensive rat, Mesenteric artery

Hypertension is the most important risk factor for cerebrovascular and cardiovascular diseases such as stroke and ischemic heart diseases. Calcium antagonists are becoming common as the first-line therapy for treatment of hypertension because of their powerful vasodilator properties. However, short-acting dihydropyridine (DHP) calcium antagonists such as nifedipine and nicardipine have been shown to cause undesirable side effects such as sympathetic activation and reflex tachycardia, thereby inducing cardiac events (1, 2). Recently, high doses (nifedipine >60 mg/day) of short-acting DHP calcium antagonists have been reported to be related to the risk of myocardial infarction and death (1, 2). To reduce these events, therefore, long-acting DHP calcium antagonists such as amlodi-

pine, barnidipine and pranidipine have been developed and widely used in the treatment of hypertension. The long-acting DHP calcium antagonists at low doses are effective in hypertension, and they have few cardiac and sympathetic side effects and also do not affect the metabolism of glucose and lipid and insulin action (3).

Sympathetic, adrenergic vasoconstrictor nerves maintain the tone of resistance blood vessels. However, recent studies have shown nonadrenergic, noncholinergic (NANC) vasodilator innervation in blood vessels and the involvement of vasodilator nerves in the regulation of vascular tone (4–7). We have demonstrated that the mesenteric resistance blood vessels of the rat are innervated by NANC vasodilator nerves in which calcitonin gene-related peptide (CGRP), a potent vasodilator neuropeptide (7), acts as the vasodilator neurotransmitter (8). CGRP-containing vasodilator nerves (CGRPergic nerves) suppress the adrenergic

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nerve function, and conversely, adrenergic nerves decrease presynaptically the function of CGRPergic nerves (9–11). Thus, we proposed that CGRPergic vasodilator nerves as well as sympathetic, adrenergic vasoconstrictor nerves regulate the tone of mesenteric resistance blood vessels.

The impairment of the control systems regulating peripheral vascular resistance is suggested as a cause of chronic hypertension because the increased total peripheral vascular resistance maintains the elevated blood pressure in chronic hypertension (12, 13). The enhanced activity of sympathetic vasoconstrictor nerves has been shown to be an important factor in the increased tone of peripheral resistance vessels in spontaneously hypertensive rats (SHR) (12, 13). We have demonstrated that the function of CGRPergic nerves in SHR decreases with age and have previously proposed that the malfunction of CGRPergic nerves plays an important role in the development and maintenance of hypertension in SHR (14, 15). However, the mechanisms underlying the reduced function of CGRPergic nerves in SHR remain unresolved. Recently, we reported that long-term treatment (7 weeks) with angiotensin-converting enzyme (ACE) inhibitors such as captopril and temocapril restored the reduced vasodilator response mediated by CGRPergic nerves in SHR (16, 17). However, little is known about the effect of the long-acting calcium antagonist on perivascular nerve function.

The present study was, therefore, undertaken to investigate further the effects of long-term treatment with long-acting calcium antagonists (amlodipine and pranidipine) on the periarterial nerve function in SHR and compare them with treatment with a short-acting calcium antagonist (nicardipine).

MATERIALS AND METHODS

Animals and long-term treatment with calcium antagonist

Male SHR at 8 weeks of age, purchased from Charles River Japan (Shizuoka), received 0.01% (10 mg/kg per day) and 0.02% (20 mg/kg per day) amlodipine or 0.1% (100 mg/kg per day) nicardipine in drinking water or 0.0035% (2 mg/kg per day) and 0.035% (20 mg/kg per day) pranidipine in rat chow. Each rat received the respective treatment for a period of 7 weeks. The non-treated control SHR received normal drinking water and rat chow. They were housed in a controlled ambient temperature of 22°C with 50 ± 10% relative humidity and a 12-h light/dark cycle.

Blood pressure measurement

The animals at 15 weeks of age were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. The left carotid artery was cannulated, and mean blood pressure (MBP) was measured with a pressure trans-

ducer (TP-200T; Nihon Kohden, Tokyo) and recorded on a pen-recorder.

Perfusion of mesenteric vascular bed

Under pentobarbital anesthesia, the mesenteric vascular bed was isolated and prepared for perfusion, as described previously (18, 19). The superior mesenteric artery was cannulated and flushed gently with Krebs-Ringer bicarbonate solution (Krebs solution) in order to eliminate blood from the vascular bed. After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The preparation was perfused with Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump (AC-2120; Atto Co., Tokyo). The preparation was also superfused with the same solution at a rate of 0.5 ml/min to prevent drying. Modified Krebs solution of the following composition was used: 120 mM NaCl, 5.0 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 0.027 mM 2NaEDTA and 11.0 mM glucose (pH 7.4). The Krebs solution was bubbled with a mixture of 95% O₂-5% CO₂ before passage through a warming coil maintained at 37°C. Changes in the perfusion pressure were measured with a pressure transducer (TP-200T) and pressure amplifier (AP-641G, Nihon Kohden) and recorded on a pen-recorder.

Periarterial nerve stimulation (PNS) and bolus injection of norepinephrine (NE) and CGRP

After allowing the basal perfusion pressure to stabilize, the preparation was subjected to PNS at 4, 8 and 12 Hz, which caused an increase in perfusion pressure due to sympathetic adrenergic vasoconstriction (20). Thereafter, the preparation was contracted with methoxamine (an α_1 -adrenoceptor agonist) at a submaximal concentration of 5 to 7 μ M in the presence of 5 μ M guanethidine, which was added to block adrenergic neurotransmission. The increased perfusion pressure was allowed to stabilize, and the preparation was again subjected to PNS at 0.5, 1, 2, 4 and 8 Hz, producing a decrease in perfusion pressure due to vasodilation mediated by CGRPergic nerves (8). All PNSs were applied for 30 s through bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1-ms duration and supramaximal voltage (50 V) were applied using an electronic stimulator (SEN 3301, Nihon Kohden).

In the perfused mesenteric preparation, NE (1, 2 and 5 nmol) and CGRP (100 and 200 pmol) were directly injected into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus, South

Natick, MA, USA). A volume of 100 μ l was injected for 10 s.

At the end of each experiment, 100 μ M papaverine was perfused through the preparation to produce complete relaxation. Vasodilation was expressed as a percentage of the maximum relaxation induced by papaverine.

Assay of norepinephrine (NE) in perfusate

In the perfused mesenteric vascular bed with resting tone, the perfusate was collected for 3 min during and after PNS (8 Hz) for 30 s. The sample was added with 50 mg activated alumina, 0.5 ml of 1 M Tris-HCl buffer (pH 9.5), and 10 μ l internal standard (I.S.; 3,4-dihydrobenzylamine, 100 ng/ml) and then stirred for 10 min. The alumina was collected in a minicolumn, and absorbed catecholamines were eluted with 400 μ l of 2% acetic acid in 0.1 mM EDTA. A 20 μ l aliquot was injected into a high-performance liquid chromatography (HPLC) apparatus to determine the concentration of NE. The HPLC system was composed of a pump (EP-300; Eicom Co., Kyoto), reverse-phase column (Eicompak CA-50DS, 2.1 $\times$ 150 mm) and an electrochemical detector (ECD-300, Eicom). The mobile phase used was 0.1 M phosphate buffer containing 600 mg/l sodium 1-octanesulfonate, 10% methanol and 50 mg/l EDTA at pH 6.0.

Statistical analyses

Data were indicated as the mean $\pm$ S.E.M. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's test were used to determine the significance of difference between values of different groups. A value of $P < 0.05$ was considered statistically significant.

Drugs

The following drugs were used: amlodipine (Sumitomo Pharmaceutical Co., Tokyo), nicardipine (Yamanouchi Pharmaceutical Co., Tokyo), pranidipine (Otsuka Pharmaceutical Co., Tokyo), NE hydrochloride (Sankyo Co., Tokyo), guanethidine sulfate (Tokyo Kasei, Tokyo), methoxamine hydrochloride (Nihon Shinyaku, Tokyo), rat CGRP (Peptide Institute, Osaka) and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka). Amlodipine and nicardipine were dissolved in drinking water at final concentrations of 0.01%, 0.02% and 0.1%, respectively. Pranidipine was mixed in rat chow at concentrations of 0.0035% and 0.035% (Oriental Yeast, Osaka; supplied by Ohtsuka Pharmaceutical Co.). All other drugs were dissolved in distilled water and diluted with Krebs solution containing 5 to 7 μ M methoxamine and 5 μ M guanethidine, when perfused and injected directly.

RESULTS

MBP of SHR following long-term treatment with calcium antagonist

As shown in Fig. 1, MBP in SHR treated with amlodipine, pranidipine and nicardipine were significantly lower than that in non-treated control SHR.

Vasoconstrictor responses to PNS and NE injection in mesenteric vascular beds long-term treated with calcium antagonist

As shown in Fig. 2, PNS (4, 8, 12 Hz) of the perfused mesenteric vascular bed with resting tone produced a frequency-dependent increase in perfusion pressure due to vasoconstriction. The pressor responses to PNS at 4 and 8 Hz were significantly lower in amlodipine- and pranidipine-treated SHR preparations than in non-treated control SHR preparations (Fig. 2). In nicardipine-treated preparations, the PNS-induced vasoconstriction was significantly smaller than in non-treated control preparations.

In perfused mesenteric vascular beds with resting tone, bolus injection of NE (1, 2 and 5 nmol) caused a concentration-dependent increase in perfusion pressure due to

Fig. 1. Mean blood pressure following 7 weeks treatment with amlodipine, pranidipine and nicardipine in spontaneously hypertensive rats (SHR). Data indicate the mean $\pm$ S.E.M. from 4–7 experiments. ** $P < 0.01$ vs non-treated control SHR.

Fig. 2. Effects of long-term treatment with amlodipine, pranidipine and nicardipine on vasoconstrictor responses to periarterial nerve stimulation (PNS) in perfused mesenteric vascular beds of spontaneously hypertensive rats (SHR). Data indicate the mean $\pm$ S.E.M. from 4–7 experiments. MPP, mean perfusion pressure. * P <0.05, ** P <0.01 vs non-treated control SHR.

Fig. 3. Effects of long-term treatment with amlodipine, pranidipine and nicardipine on vasoconstrictor responses to bolus injection of norepinephrine (NE) in perfused mesenteric vascular beds of spontaneously hypertensive rats (SHR). Data indicate the mean $\pm$ S.E.M. from 4–7 experiments. MPP, mean perfusion pressure.

vasoconstriction. There was no significant difference between amlodipine- and pranidipine-treated SHR preparations and non-treated control SHR preparations (Fig. 3). The NE (5 nmol)-induced vasoconstriction in nicardipine-treated SHR preparations was smaller than that in non-treated SHR preparations (Fig. 3), but there was no significant difference between treated SHR and non-treated SHR.

NE overflow evoked by PNS in mesenteric vascular beds long-term treated with calcium antagonists

The PNS (8 Hz) of perfused mesenteric vascular beds with resting tone evoked overflow of NE in the perfusate. The PNS-evoked NE overflow was significantly less in am-

lodipine- and pranidipine-treated SHR preparations than in non-treated control SHR preparations (Fig. 4). However, the PNS-evoked NE overflow in nicardipine-treated SHR preparations was significantly greater than that in non-treated control SHR preparations (Fig. 4).

Vasodilator responses to PNS and CGRP injection in mesenteric vascular beds long-term treated with calcium antagonists

To maintain an active tone of the perfused mesenteric bed, the preparation was constricted by continuous perfusion of 5 to 7 μ M methoxamine in the presence of 5 μ M guanethidine. In the preparation with active tone, PNS (0.5 to 8 Hz) produced a frequency-dependent decrease in per-

Fig. 4. Effects of long-term treatment with amlodipine, pranidipine and nicardipine on norepinephrine (NE) overflow evoked by periarterial nerve stimulation (PNS, 8 Hz) in perfused mesenteric vascular beds of spontaneously hypertensive rats (SHR). Data indicate the mean $\pm$ S.E.M. from 4–7 experiments. * P <0.05, ** P <0.01 vs non-treated control SHR.

Fig. 5 Effects of long-term treatment with amlodipine, pranidipine and nicardipine on vasodilator responses to periarterial nerve stimulation (PNS) in perfused mesenteric vascular beds of spontaneously hypertensive rats (SHR) with active tone. Data indicate the mean $\pm$ S.E.M. from 4–7 experiments.

fusion pressure due to vasodilation. As shown in Fig. 5, there was no significant difference in the PNS-induced vasodilation between each of the calcium antagonist-treated SHR preparations and the non-treated control SHR preparations (Fig. 5).

In the perfused mesenteric vascular bed with active tone, bolus injection of CGRP (100 and 200 pmol) induced a concentration-dependent decrease in perfusion pressure due to vasodilation. There was no significant difference in CGRP-induced vasodilation between each calcium antagonist-treated SHR preparation and non-treated control SHR preparations (data not shown).

DISCUSSION

The present study demonstrated that PNS of perfused mesenteric vascular beds with resting tone in SHR resulted in a frequency-dependent increase in perfusion pressure due to vasoconstriction and overflow of NE in the perfusate. This vasoconstriction induced by PNS has been shown to be abolished by the neurotoxin tetrodotoxin (TTX), adrenergic neuron blocker guanethidine, and the α -adrenoceptor antagonist prazosin (20, 21). Also, the PNS-evoked NE overflow in the perfusate has been shown to be sensitive to TTX and guanethidine and calcium removal from the medium (22). These findings indicate that the

PNS-induced vasoconstriction is mediated by endogenous NE released from sympathetic adrenergic nerve terminals. In the present study, long-term treatment of SHR with calcium antagonists (amlodipine, pranidipine and nicardipine) inhibited the PNS-induced vasoconstriction in the perfused mesenteric vascular preparations. Both amlodipine and pranidipine treatments markedly reduced the PNS-induced NE release in the perfusate, whereas nicardipine treatment significantly enhanced the NE overflow. Since both amlodipine and pranidipine did not inhibit vasoconstrictor responses to exogenously applied NE, it is likely that the vasoconstriction in response to adrenergic stimulation is due to decreased NE release from the nerve terminals. Recently, the L-type Ca^{2+} channel antagonist of DHP derivatives such as cilnidipine has been reported to inhibit N-type Ca^{2+} channels in the nerve terminal and decreases transmitter release evoked by nerve stimulation (23, 24). The N-type but not L-type Ca^{2+} channels are involved in neurotransmitter release (25–27). Amlodipine has been shown to have a blocking effect on N-type Ca^{2+} channels (28). Therefore, reduction of the PNS-evoked NE release after long-term treatment with amlodipine or pranidipine may be due to inhibition of N-type Ca^{2+} channels. However, nicardipine treatment inhibited the neurogenic vasoconstriction and tended to decrease the vasoconstriction in response to bolus injection of NE. Since nicardipine treatment markedly increased the PNS-evoked NE release, it appears likely that postsynaptic α -adrenoceptors may be down-regulated by excess NE release from the nerve terminals. This may cause reduced vasoconstrictor responses to PNS, even though PNS enhances NE release.

The present study, in agreement with previous studies (8–10), showed that PNS of SHR perfused mesenteric vascular beds with active tone produced by methoxamine in the presence of guanethidine results in a frequency-dependent decrease in perfusion pressure due to vasodilation. This vasodilation is sensitive to TTX and the CGRP-receptor antagonist CGRP(8–37). In addition, PNS evokes the release of CGRP (8, 29), indicating that this PNS-induced vasodilation is mediated by endogenous CGRP released from CGRPergic nerve terminals. We reported that both CGRPergic nerve-mediated vasodilation and PNS-induced CGRP release in SHR were smaller than in normotensive Wistar Kyoto rats (WKY) and both phenomena decrease with age (14, 15, 30). Moreover, it was previously reported that the mesenteric arteries of 15 and 30-week-old SHR have a lower density of CGRP-containing fibers compared with age-matched WKY and 8-week-old SHR (14, 15). Taken together, this evidence suggests that the function of CGRPergic vasodilator nerves in the mesenteric resistance blood vessel of SHR decreases with age. In the present study, the long-term treatment of SHR with each calcium antagonist used did not affect the PNS-induced vasodila-

tion. The present findings are in accord with the previous study that long-term treatment of SHR with nicardipine had no effect on the PNS-induced vasodilation (16). Furthermore, these calcium antagonists were without effect on the vasodilation induced by exogenous CGRP. The DHP calcium antagonist, nifedipine, has been shown to have no effect on neurogenic CGRP release from CGRPergic nerve terminals (31). Furthermore, it has been reported that the N-type Ca^{2+} channel is not involved in the neurogenic CGRP release from nerve terminals of the rat ureter (32). Therefore, it is likely that long-term treatment with calcium antagonists does not affect the CGRPergic nerve function.

The PNS of the rat mesenteric artery can evoke activation of both adrenergic and CGRPergic nerves (9–11). The inhibition of CGRPergic nerve function by the CGRP receptor antagonist (CGRP(8–37)) and capsaicin (depletor of CGRPergic nerves) potentiated the vasoconstriction induced by adrenergic nerve stimulation (10, 11). We reported that CGRPergic nerves have an ability to suppress the adrenergic nerve-mediated vasoconstriction. In the present study, nicardipine treatment reduced the adrenergic vasoconstriction in response to PNS, but it enhanced the release of transmitter NE. Since the calcium antagonists used did not affect the CGRPergic nerve function, the reduced vasoconstriction in response to PNS after nicardipine treatment may have resulted from the counteraction due to CGRPergic nerve activation in addition to down-regulated postsynaptic α -adrenoceptors. The counteraction of CGRPergic nerves against adrenergic nerve-mediated vasoconstriction may also be involved in the inhibition of the PNS-induced vasoconstriction after treatment with amlodipine and pranidipine.

In conclusion, the present results suggest that long-term treatment of SHR with the long-acting calcium antagonists, amlodipine and pranidipine, inhibit the function of periarterial sympathetic nerves by reducing the release of neurotransmitter NE, whereas the short acting calcium antagonist, nicardipine, facilitates transmitter release from adrenergic nerve terminals. It is also suggested that long-term treatment with calcium antagonists has no effect on CGRPergic nerve function.

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