Original Article

Continuous Blockade of L-Type Ca\(^{2+}\) Channels Suppresses Activation of Calcineurin and Development of Cardiac Hypertrophy in Spontaneously Hypertensive Rats

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We examined whether Ca\(^{2+}\) channel blockers inhibit the activation of the Ca\(^{2+}\)-dependent phosphatase calcineurin and the development of cardiac hypertrophy in spontaneously hypertensive rats (SHR). We randomly divided 12-week-old SHR into three groups, one each receiving vehicle, bolus injection or continuous infusion of nifedipine (10 mg/kg/day) from 12 to 24 weeks of age. Systolic blood pressure (BP) and heart rate were measured every week after the treatment using the tail-cuff plethysmography method. After 4, 8 and 12 weeks of treatment, 6 rats of each group were subjected to examinations that included an assay for calcineurin activity in the heart, magnetic resonance imaging (MRI), histology and Northern blot analysis. Continuous infusion of nifedipine consistently reduced BP, whereas bolus injection resulted in a fluctuation of BP. Continuous infusion of nifedipine not only reduced left ventricular mass but also decreased the transverse diameter of cardiomyocytes, interstitial fibrosis and the expression of the atrial natriuretic peptide and brain natriuretic peptide genes in the heart, while bolus injection of nifedipine did not significantly attenuate any of these hypertrophic responses in SHR. The activity of calcineurin in the heart was strongly suppressed by continuous but not bolus infusion of nifedipine in SHR. The results indicate that continuous blockade of Ca\(^{2+}\) channels with nifedipine effectively suppresses the development of cardiac hypertrophy in SHR, possibly through inhibition of the calcineurin activity. (Hypertens Res 2002; 25: 117 – 124)

Key Words: Ca\(^{2+}\), calcineurin, cardiac hypertrophy, magnetic resonance imaging, nifedipine

Introduction

Cardiac hypertrophy is not only an adaptive response of the heart to pressure overload but also a leading predictor of progressive heart disease and morbidity (1). Epidemiological studies have shown that cardiac hypertrophy causes ischemic heart disease, arrhythmia and sudden death. It is thus of paramount importance to elucidate the mechanisms of development of cardiac hypertrophy. Since cardiac myocytes lose their proliferative ability after birth, the development of cardiomyocyte hypertrophy is the only available means of re-
hypertrophy (some rodent models of pressure overload-induced cardiac cyclosporin A or FK506 prevents hypertrophic responses in hypertensive rats (SHR).

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tion of calcineurin (16). Intracellular Ca\(^{2+}\) levels are also elevated by various hypertrophic stimuli (4) and Ca\(^{2+}\) has been reported to play a critical role in the development of cardiac hypertrophy (5). Activation of the Ca\(^{2+}\)-dependent protein phosphatase calcineurin induces cardiac hypertrophy, and the development of hypertrophy of cultured cardiomyocytes in response to angiotensin II or phenylephrine is inhibited by calcineurin inhibition such as cyclosporin A and FK506 (5). Furthermore, cyclosporin A or FK506 prevents hypertrophic responses in some rodent models of pressure overload-induced cardiac hypertrophy (6–9).

Ca\(^{2+}\) channel blockers are effective to reduce blood pressure (10, 11). Among them, nifedipine is a prototype of the dihydropyridine Ca\(^{2+}\) channel blockers, and is widely used in the treatment of essential hypertension (11). Although many reports have indicated that short-acting nifedipine has unfavorable effects on hypertensive patients with cardiac hypertrophy (12, 13), long-acting nifedipine may induce regression of left ventricular hypertrophy in hypertensive animals and patients (14, 15). Since Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels has been reported to be important for activation of calcineurin (16), we here examined whether nifedipine can suppress the activation of calcineurin and prevent the development of cardiac hypertrophy in spontaneously hypertensive rats (SHR).

To examine whether long-acting nifedipine and short-act-

Materials and Methods

Twelve-week-old male SHR were obtained from Charles River Japan (Tokyo, Japan). The animals were randomly divided into three separate groups receiving: 1) intravenous injection of vehicle (96% ethanol : polyethylene glycols 400 : water = 15 : 15 : 70) three times a day through the right jugular vein; 2) i.v. injection of nifedipine (Bayer Yakuhin, Ltd., Tokyo, Japan) (10 mg/kg/day) three times a day through the right jugular vein; 3) continuous infusion of nifedipine (10 mg/kg/day, 0.5 µl/h) by a subcutaneously implanted osmotic minipump (Alzet\(^{\circledR}\), model 2002; Muromachi Kitai Co., Ltd., Tokyo, Japan). All treatments were started from the age of 12 weeks, when cardiac hypertrophy had already developed (17), and continued to 24 weeks (Fig. 1). Systolic blood pressure (BP) and heart rate (HR) were measured before treatment and every week after administration of vehicle or nifedipine using the tail-cuff plethysmography method in conscious rats (17). After 4, 8 and 12 weeks of treatment, 6 rats of each group were subjected to the following analyses. All protocols were approved by the guidelines of the University of Tokyo.

Magnetic Resonance Imaging (MRI) Analysis

We used MRI (Varian NMR imaging spectrometer UNITY Plus 300; Varian Associates, Inc., Palo Alto, USA) to assess left ventricular (LV) mass in the present study. MRI studies of rat LV mass were performed as described previously (18). Sodium pentobarbital (25 mg/kg) was used for general anesthesia during the MRI study. A magnet with a field strength of 1.0-T units was used for imaging in the protocol. Gated short-axis paracoronal spin-echo views of the heart were obtained at a repetition time of ≥ 300 ms as determined by
with the size-fractionated in 1.2% formaldehyde agarose gels and (Cinna Biotecx Laboratories, Inc.). Ten µg of total RNA was size-fractionated in 1.2% formaldehyde agarose gels and transferred to nylon membranes. The blots were hybridized with the [α-32P]dCTP (Du Pont-New England Nuclear Co., Boston, USA)-labeled cDNA fragments of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (8). The hybridized bands were quantitated by a densitometer.

Histological Analysis
Hearts from SHR were fixed with 10% formalin by perfusion fixation, embedded in paraffin and sectioned at 4 µm thickness. The sections were stained by hematoxylin-eosin (H-E) and van Gieson to evaluate cardiomyocyte size and myocardial fibrosis, respectively (8, 17). The transverse diameter of cardiomycytes was measured by micrometers at 20 randomly chosen points from a cross section of the LV myocardial free wall (17). The extent of LV fibrosis was measured in 8 randomly selected fields from a section by calculating the ratio of the van Gieson-stained fibrotic area to the total myocardial area (8).

Northern Blot Analysis
Total RNA of LV tissue was extracted using RNA zol B (Cinna Biotecx Laboratories, Inc.). Ten µg of total RNA was size-fractionated in 1.2% formaldehyde agarose gels and transferred to nylon membranes. The blots were hybridized with the [α-32P]dCTP (Du Pont-New England Nuclear Co., Boston, USA)-labeled cDNA fragments of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (8). The hybridized bands were quantitated by a densitometer.

Table 1. BP in SHR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BP (mmHg)</th>
<th>Vehi</th>
<th>B-Ni</th>
<th>C-Ni</th>
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<tr>
<td>0 week</td>
<td>0 h</td>
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<tr>
<td>8 week</td>
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<td>4 h</td>
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<tr>
<td>12 week</td>
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All treatments were started from 12 weeks of age (= 0 week). The BP of SHR treated with bolus injection of vehicle (Vehi) and nifedipine (B-Ni) for 8 and 12 weeks was recorded at 0, 1 and 4 h after injection. The BP of rats treated with continuous infusion of nifedipine (C-Ni) for 8 and 12 weeks was measured at the same time. Each value represents the mean ± SEM of 6 rats. *p < 0.05 vs. 0 h BP of vehicle treated SHR of same age. **p < 0.05 vs. 0 h BP of SHR of the same age treated by bolus injection of nifedipine.

Calcineurin Activity
The activity of calcineurin was determined using phosphorylated GST-RII peptide as a substrate according to the previously described method (8) with some modifications. We separated calmodulin-bound calcineurin (active calcineurin, molecular weight > 100 kDa) from free calcineurin (inactive calcineurin, molecular weight < 100 kDa) by centrifugation of cell lysates at 1,500 g for 10 min using Ultrafree-MC Centrifugal Filter Units (Millipore, Bedford, USA). The phosphorylated GST-RII peptide was incubated with the samples under the Ca2+-free condition.

Statistical Analysis
All values are expressed as the mean ± SE of six SHR in each instance. Differences between experimental groups were evaluated for statistical significance by one-way ANOVA followed by Dunnet’s modified t-test. Values of p < 0.05 were considered to indicate statistical significance.

Results

Changes of BP and HR
Treatment of SHR with vehicle or nifedipine was started from 12 weeks after birth (Fig. 1). Treatment with nifedipine significantly reduced BP (Table 1). There was no significant difference in BP between the two nifedipine-treatment groups (Table 1, 8 week 0 h, 12 week 0 h). When nifedipine was infused intravenously, however, the BP was transiently decreased at 1 h after infusion and returned to the basal levels at 4 h, while BP was constant when nifedipine was con-
tinuously infused by an osmotic pump (Table 1). These results indicate that BP fluctuated markedly in rats treated with a bolus injection of nifedipine as compared with the relatively steady change in BP in rats treated with continuous infusion of nifedipine. There was no significant difference in basal HR among the three groups of SHR (Table 2). However, the HR recorded at 1 h after i.v. injection of nifedipine was faster than that in SHR treated continuously with nifedipine (Table 2).

**Regression of Cardiac Hypertrophy by Nifedipine**

MRI was used to assess LV mass in SHR (Fig. 2A). There was no significant difference in body weight among the three groups (data not shown). SHR treated with vehicle for 4, 8 and 12 weeks displayed a progressive increase in LV mass compared with 12-week-old SHR before treatment (Fig. 2B). Although there was no significant difference in LV mass between SHR at 4 weeks after treatment with nifedipine and vehicle, continuous infusion of nifedipine for 8 or 12 weeks induced a significant decrease in LV mass compared with the vehicle treatment. Treatment by bolus injection of nifedipine did not prevent the development of cardiac hypertrophy (Fig. 2B). We also examined the LV weight and the ratio of LV weight to body weight, and the results were consistent with those of the MRI (data not shown).

The transverse diameter of cardiac myocytes became larger, and LV perivascular and interstitial fibrosis became more prominent with age in vehicle-treated SHR (Fig. 3). Continuous infusion of nifedipine for 12 weeks significantly inhibited the increase in cardiomyocyte size (Fig. 3A, B) and perivascular and interstitial fibrosis (Fig. 3A, C), whereas there were no significant differences in the diameter of cardiomyocytes or myocardial fibrosis between rats treated by bolus injection of nifedipine and vehicle (Fig. 3B, C). These results suggest that continuous blockade of Ca\(^{2+}\) channels suppresses the development of cardiac hypertrophy in SHR.

**Suppression of the ANP and BNP Gene Expression by Nifedipine**

Induction of specific gene expressions is one of the hypertrophic responses to hemodynamic overload (2, 3). We therefore examined the expression of the ANP and BNP genes that have been reported to be upregulated in the hypertrophied heart (8). The mRNA levels of ANP and BNP were significantly increased in the heart after 12 weeks of treatment with vehicle as compared with those of 12-week-old
Continuous infusion of nifedipine for 12 weeks significantly suppressed the increase in ANP or BNP expressions, while upregulation of ANP and BNP gene expressions was not inhibited in the heart of SHR treated by bolus injection of nifedipine for 12 weeks (Fig. 4).

**Inhibition of Calcineurin Activity by Nifedipine**

The \( \text{Ca}^{2+} \) dependent phosphatase calcineurin has been reported to play a critical role in the development of cardiac hypertrophy (5–9). We therefore examined whether nifedipine treatment inhibits the activation of calcineurin in the heart of SHR. The calcineurin activity in the heart was significantly increased with age in vehicle-treated SHR (Fig. 5). Although treatment by bolus injection of nifedipine did not affect the progressive increase in the calcineurin activity, treatment by continuous injection of nifedipine for 8 and 12 weeks significantly suppressed the increase in the calcineurin activity in the heart (Fig. 5).

**Discussion**

Although short-acting \( \text{Ca}^{2+} \) channel blockers have been reported not to prevent the development of cardiac hypertrophy (12, 13), long-acting \( \text{Ca}^{2+} \) channel blockers may be effective to prevent or regress cardiac hypertrophy (14, 15). In the present study, although bolus injection as well as continuous infusion of nifedipine effectively reduced BP, only continuous infusion of nifedipine attenuated increases in LV mass, cardiomyocyte size, myocardial fibrosis and expres-
When nifedipine was infused intravenously, BP levels fluctuated and heart rate was increased, suggesting that the fluctuation in BP may stimulate the sympathetic nervous system. It has been reported that activation of the sympathetic nervous system activates the renin-angiotensin system (19) and that norepinephrine and angiotensin II are potent inducers of cardiac hypertrophy (17, 20–23). Therefore, the unfavorable effect of short-acting Ca\(^{2+}\)-channel blockers on hypertension-induced cardiac hypertrophy may be due to the activation of these neurohormonal factors. Long-acting nifedipine has moderate and persistent BP-lowering effects (14, 15). Long-acting Ca\(^{2+}\)-channel blockers have been reported not to increase plasma levels of catecholamines, renin and aldosterone (24). This may be one reason why long-acting nifedipine is more effective than short-acting nifedipine in preventing cardiac hypertrophy.

Calcineurin is a Ca\(^{2+}\)-dependent protein phosphatase and its activation is regulated by prolonged elevation of intracellular Ca\(^{2+}\) levels (25). A large increase in intracellular Ca\(^{2+}\) levels is induced by Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels and following Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores (4). Recently, calcineurin has been reported to play a key role in the development of cardiac hypertrophy (5). Two kinds of transgenic mice which overexpress constitutively active calcineurin and transcription factor nuclear factor of activated T cells-3 (NFAT3) have been shown to develop marked cardiac hypertrophy (5). Calcineurin inhibitors such as cyclosporin A and FK506 suppress phenylephrine- and
angiotensin II-induced cardiomyocyte hypertrophy in vitro (5) and pressure overload-induced cardiac hypertrophy in vivo (6–9). In this study, the calcineurin activity progressively increased in the heart of SHR with age, accompanied by the development of cardiac hypertrophy, suggesting that activation of calcineurin is associated with cardiac hypertrophy in SHR. Several groups have reported that cyclosporin A and FK506 do not prevent pressure overload-induced cardiac hypertrophy and that the activity of calcineurin is not increased in the overloaded heart (26–29). The reason for the discrepancy is not clear at present; however, many of these studies did not examine the activity of calcineurin in the heart before and after administration of calcineurin inhibitors (6, 7, 26, 27). It is therefore unknown whether the activities of calcineurin in the heart were effectively suppressed by calcineurin inhibitors in these experiments. It is difficult to determine the precise activity of calcineurin (30). In the present study, we measured the calcineurin activity after separating activated calcineurin from non-activated calcineurin, which enabled us to accurately determine the endogenous calcineurin activity. Our data revealed that continuous infusion of nifedipine is sufficient to suppress the activation of calcineurin in the heart of SHR, just as cyclosporin A did in pressure-overloaded rats (8).

Dihydropyridine Ca²⁺ channel blockers inhibit Ca²⁺ influx through L-type Ca²⁺ channels and then block Ca²⁺-induced Ca²⁺ release from intracellular Ca²⁺ stores, which play an important role in the large increase in intracellular Ca²⁺ levels in cardiac myocytes (31). In the present study, continuous infusion but not bolus injection of nifedipine significantly inhibited the activation of calcineurin in the heart of SHR, suggesting that long-acting but not short-acting Ca²⁺ channel blockers effectively block the Ca²⁺ influx through L-type Ca²⁺ channels and the elevation of intracellular Ca²⁺ levels in cardiac myocytes of SHR. The reflective activation of the sympathetic nervous system and the renin-angiotensin system may also contribute to the increase in calcineurin activity in the heart of SHR treated by bolus injection of nifedipine, since we and others have observed that catecholamines and angiotensin II are strong stimulators for calcineurin (unpublished data, 5). Since the activation of calcineurin as well as the development of cardiac hypertrophy were inhibited in the heart of SHR treated with continuous infusion of nifedipine, suppression of the calcineurin activity may be a cause of Ca²⁺ channel blocker-induced regression of cardiac hypertrophy. In addition to inhibition of the L-type Ca²⁺ channel function, however, we cannot ignore the BP-lowering effect of long-acting nifedipine on regression of cardiac hypertrophy. Further studies are necessary to elucidate whether other antihypertensive agents, such as ACE inhibitors, diuretics or β blockers, inhibit the development of cardiac hypertrophy and the activity of calcineurin in the heart to the same extent as a Ca²⁺ channel blocker. Calcineurin dephosphorylates and activates the NFAT3 (5). NFAT3 interacts with GATA4 and activates transcription of cardiac genes, including ANP and BNP (5). In the present study, continuous blockade of Ca²⁺ channels with nifedipine inhibited the upregulation of ANP and BNP genes in the heart of SHR. The upregulation of these genes may also be inhibited by the inhibition of calcineurin activation in the heart of SHR.

Ca²⁺/calmodulin (CaM) modulates many molecules through activation of functional molecules such as calcineurin and Ca²⁺/CaM-dependent protein kinases (CaMK). Recently, it was reported that activated CaMK induce hypertrophic responses in cardiomyocytes in vitro and in vivo, and that the transcription factor MEF2 is a downstream target for CaMK signaling in the hypertrophic heart. Therefore, there is a possibility that long-acting nifedipine inhibits the activities of CaMK as well as of calcineurin.

In conclusion, this study demonstrates that continuous blockade of Ca²⁺ channels effectively suppresses the development of cardiac hypertrophy in SHR, possibly through the inhibition of calcineurin activation in the heart, and suggests the effectiveness of long-acting Ca²⁺ channel blockers for the treatment of cardiac hypertrophy.

References

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