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Ca²⁺ channel blocker benidipine promotes coronary angiogenesis and reduces both left-ventricular diastolic stiffness and mortality in hypertensive rats

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Abstract

Background—The beneficial cardiac effects of some Ca²⁺ channel blockers have been attributed to blood pressure reduction, but these pleiotropic effects require further investigation. We compared the effects of benidipine, which has beneficial cardiac effects, and nitrendipine, which does not, in an animal model of hypertensive diastolic heart failure (DHF).

Methods and results—Male Dahl salt-sensitive rats were fed a high-salt diet from age 7 weeks to induce hypertension and were either vehicle or orally administered benidipine (3 mg/kg daily) or nitrendipine (10 mg/kg daily) from age 10 to 18 weeks. Control rats were maintained on a low-salt diet. In vehicle-treated rats, left-ventricular (LV) fractional shortening was preserved but LV end-diastolic pressure was increased, indicative of DHF. Benidipine and nitrendipine had similar antihypertensive effects and reduced both LV weight and cardiomyocyte hypertrophy. Benidipine reduced LV diastolic stiffness and mortality to a greater extent than did nitrendipine. Benidipine, but not nitrendipine, also reduced lung weight. The extent of interstitial fibrosis and the abundance of mRNAs for prohypertrophic, profibrotic, or proinflammatory genes in the left ventricle were reduced by benidipine and nitrendipine. Benidipine, but not nitrendipine, increased capillary density and restored the expression of hypoxia-inducible factor 1 α , vascular endothelial growth factor, and endothelial nitric oxide synthase in the left ventricle.

Conclusions—Benidipine reduced LV diastolic stiffness and increased survival, effects likely attributable predominantly to promotion of coronary angiogenesis rather than to attenuation of

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interstitial fibrosis. Benidipine may thus be more effective than purely L-type Ca^{2+} channel blockers in preventing hypertensive DHF.

Keywords

angiogenesis; cardiac stiffness; diastolic heart failure; hypoxia-induced factor- α ; L-type Ca^{2+} channel

Introduction

Chronic pressure overload due to hypertension results in myocardial hypertrophy as an adaptive response to maintain cardiac function. However, persistent left-ventricular hypertrophy (LVH) leads to diastolic heart failure (DHF) [1,2], which accounts for 30–50% of all cases of heart failure and has a poor prognosis [3,4]. DHF is characterized by abnormal LV relaxation, impaired LV filling, and increased LV diastolic stiffness, with LV systolic function remaining largely unaffected.

Given that increased interstitial fibrosis has been associated with the onset of DHF [5], agents that block the renin–angiotensin–aldosterone system are used for the treatment of this condition [6]. However, given that treatment with a single antihypertensive angiotensin-converting enzyme inhibitor (ACEI) or angiotensin-receptor blocker (ARB) is often insufficient to normalize blood pressure, calcium channel blockers (CCBs) are often used in combination with ARBs or ACEIs in the clinical setting [7]. In addition to ACEI and ARB, recent few studies in experimental animals have shown that CCBs are also effective in preventing cardiac remodeling and dysfunction [8]. In fact, it has been reported that CCBs exert cardiovascular protective actions including reduction of oxidative stress and proinflammatory response [2,9]. However, the precise mechanisms underlying the cardioprotection afforded by a CCB in animals or patients with hypertensive DHF remain largely unknown.

The physiological role of T-type Ca^{2+} channel is diverse, and clinical advantages of T-type channel blockade were assessed in late 1990s using mibefradil, another class Ca^{2+} channel antagonist. The drug had relatively selective blocking action on T-type Ca^{2+} channel, and beneficial effects were shown in the treatment of cardiac hypertrophy, angina pectoris and renal failure [10–12]. However, mibefradil, which is known to block all three subtypes of T-type Ca^{2+} channel (α_{1G} , α_{1H} and α_{1I}) [13], was abandoned for clinical usage because of frequent drug–drug interaction. In addition, some studies show that some dihydropyridines (DHPs) have blocking action T-type Ca^{2+} channels in native tissues [14,15]. As subtype-specific blocking action of DHPs was not assessed in these studies, different expression levels of each T-type channel subtype would result in different blocking action in case that DHP had subtype-specific blocking action.

Recent study has indicated that DHP Ca^{2+} antagonists have a different profile in blocking T-type Ca^{2+} channel subtypes expressed in *Xenopus* oocytes [16,17]. Several studies showed beneficial effect of efonidipine on renal function, cardiac failure and aldosterone secretion as a result of the T-type Ca^{2+} channel-blocking action of the drug [18–20]. Accumulating evidence suggested that up-regulation of T-type Ca^{2+} channels has been associated with both LVH and hypertensive DHF [21,22]. To elucidate the cardiac protective effects as well as the mechanism of action of benidipine against the activated T-type Ca^{2+} channel, we compared the effects of benidipine, a blocker of T-type and L-type Ca^{2+} channels, with those of nitrendipine, a blocker of L-type Ca^{2+} channel, in a Dahl salt-sensitive rat model of hypertensive DHF.

Methods

Animals

Male inbred Dahl salt-sensitive rats were obtained from Japan SLC (Hamamatsu, Japan) and were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996). Weaning rats were fed laboratory chow containing 0.3% NaCl until 7 weeks of age. Animals on this latter diet served as models of hypertensive LVH at 10 weeks of age and DHF at 18 weeks of age [1]. Both food and tap water were provided *ad libitum* throughout the experiment. The rats on the high-salt diet were divided into three groups: those orally given benidipine (3 mg/kg body weight daily; Kyowa Hakko Kirin CO., Ltd, Tokyo, Japan) from 10 to 18 weeks of age ($n = 10$); those orally given nitrendipine (10 mg/kg daily; Sigma Aldrich) from 10 to 18 weeks of age ($n = 10$); and those orally given vehicle (0.5% carboxymethylcellulose) from 10 to 18 weeks of age ($n = 10$, untreated group). The benidipine, nitrendipine, and vehicle were each given by oral gavage every day. Rats maintained on the 0.3% NaCl diet until 18 weeks of age were studied as a control group ($n = 10$). At 18 weeks of age, rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and were subjected to hemodynamic and echocardiographic analyses. The heart and kidney were subsequently excised, and LV and kidney tissues were stored at -80°C for either molecular analyses, or fixed with paraformaldehyde for pathological analysis.

Echocardiographic and hemodynamic analyses

Systolic blood pressure was measured weekly in conscious animals by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) [23]. At 18 weeks of age, rats were subjected to transthoracic echocardiography as previously described [23]. Echocardiography was performed with a SONOS 7500 ultrasound system and an ultraband transducer of 5–12 MHz (Philips, Andover, Massachusetts, USA). LV end-diastolic (LVDd) and end-systolic (LVDs) dimensions as well as the thickness of the inter-ventricular septum (IVST) were measured. LV fractional shortening was calculated as $100\% \times (\text{LVDd} - \text{LVDs})/\text{LVDd}$. After echocardiography, a 2F micromanometer-tipped catheter (SPR-407; Millar Instruments, Houston, Texas, USA) that had been calibrated relative to atmospheric pressure was inserted through the right carotid artery into the left ventricle. We evaluated the maximum first derivative of LV pressure ($\text{LV } dP/dt_{\text{max}}$) as an index of contractility, minimal rate of LV pressure change ($\text{LV } dP/dt_{\text{min}}$), and the pressure half-time ($T_{1/2}$) as an index of relaxation. Tracings of LV pressure and the electrocardiogram were digitized to determine the pressure half-time and LV end-diastolic pressure as previously described [2].

Histology

The left ventricle was fixed with ice-cold 4% paraformaldehyde for 16–24 h, embedded in paraffin, sectioned transversely (thickness 3 μm), and stained either with hematoxylin–eosin for evaluation of cardiomyocyte hypertrophy or with Azan-Mallory solution for evaluation of interstitial fibrosis. The cross-sectional areas of cardiomyocytes and the areas of the fibrosis in the interstitial region were calculated in 10 randomly chosen microscopic fields from three different sections in each animal, as previously described [24,25]. The sections were also immunostained with mouse monoclonal antibody to rat CD31 (1 : 100 dilution; Pharmingen, San Diego, California, USA), and a Universal Immuno-Enzyme Polymer kit (Nichirei Biosciences, Tokyo, Japan) was used to visualize the coronary capillary endothelial cells. The capillary endothelial cells were quantified by measuring the number of CD31⁺ cells per high-power field (400 $\times$). The number of capillaries was measured in 15 randomly chosen microscopic fields from three different sections in each animal. The evaluation of capillary diameter was also analyzed in each high-power field (400 $\times$).

Quantitation of gene expression

Total RNA was extracted from LV tissue, and the abundance of specific mRNAs was determined by reverse transcription and real-time polymerase chain reaction (PCR) analysis with a Prism 7700 Sequence Detector (Perkin-Elmer, Waltham, Massachusetts, USA). The sequences of primers and TaqMan probes specific for β -myosin heavy chain (β -MHC), angiotensin-converting enzyme (ACE), transforming growth factor (TGF)- 1β , or collagen types I or III have been described previously [25], as have been those for vascular endothelial growth factor (VEGF), VEGF receptor-1 (Flt-1), hypoxia-inducible factor (HIF)- 1α , and endothelial nitric oxide synthase (eNOS) [24,25]. PCR was also performed with oligonucleotides specific for the α 1G subunit of T-type Ca^{2+} channels (5'-CCTGCCTGTTGCCGAGAG-3', 5'-CTGTCTGTGTTACTGGATTCCTTCC-3', and 5'-AGATTCCTGGTCGGCCTATATCTTTCC-3' as the forward primer, reverse primer, and TaqMan probe, respectively; GenBank accession no. AF027984). TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents (Applied Biosystems, Foster City, California, USA) were used to detect GAPDH mRNA as an internal standard.

Immunoblot analysis

Tissue samples (80 μ g of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, California, USA). The membrane was incubated at room temperature, first for 1 h with Tris-buffered saline containing 5% nonfat milk and 0.1% Tween-20 and then overnight with rabbit polyclonal antibodies to VEGF-A, to VEGF-C (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), to HIF- 1α (Novus Biologicals, Littleton, Colorado, USA), to Phospho-eNOS (p-eNOS, Ser-1177), or to eNOS (both from Cell Signaling, Danvers, Massachusetts, USA), all at a 1 : 1000 dilution in the same solution. The membrane was washed and then incubated at room temperature for 1 h with a 1 : 1000 dilution of horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (MBL, Nagoya, Japan), after which immune complexes were detected and quantified as described previously [24]. The intensities of the VEGF, HIF- 1α , and eNOS bands were quantified by densitometry with ATTO CS Analyzer (version 1.01) software, and the amount of each protein was normalized against that of GAPDH determined with rabbit antibodies to this protein (Santa Cruz Biotechnology, Inc.).

Statistics

Data are presented as means $\pm$ SEM. Differences in various parameters between the four groups were evaluated by analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Survival rate was analyzed by the standard Kaplan–Meier method with a log-rank test. A *P* value of less than 0.05 was considered statistically significant.

Statement of responsibility

The authors had full access to the data and take responsibility for data integrity. All authors have read and agree to the manuscript as written.

Results

Systolic blood pressure

Dahl salt-sensitive rats fed a high-salt diet from 7 weeks of age progressively developed hypertension [1,2]. Treatment with benidipine or nitrendipine from 10 weeks of age similarly lowered systolic blood pressure by approximately 15–20 mmHg in the conscious

state, with this effect being apparent as early as 1 week after the initiation of treatment (Fig. 1, Table 1).

Survival rate

Kaplan–Meier analysis revealed that the survival rate of rats in the untreated group up to 18 weeks of age was half that of animals in the control group (no deaths). The survival rate was increased slightly in the nitrendipine group (60%) compared with that in the untreated group (50%), but no deaths were observed in the benidipine group (Fig. 2).

Left-ventricular geometry and function

Heart rate was greater in the untreated group than in the control group at 18 weeks of age, although this increase was not significant. Heart rate was significantly reduced by treatment with benidipine compared with that in the untreated and nitrendipine groups (Table 1). Both the thickness of the IVST and the ratio of LV weight to body weight were significantly greater in the untreated group than in the controls, indicative of LVH; both of these effects were significantly reduced by nitrendipine or benidipine. LV fractional shortening did not differ among the four experimental groups. The LV $dP/dt_{\max}$ was lower in the untreated group than in the control group; this change was improved by benidipine and nitrendipine. The LV dP/dt was smaller, whereas the $T_{1/2}$ was greater in the untreated group than in the controls. The changes in these parameters were also improved by both treatments.

The LV end-diastolic pressure and the ratio of lung weight to body weight were greater in the untreated group than in the control group. Nitrendipine significantly reduced the extent of both parameters, whereas benidipine prevented it. In addition, the weight of the right-ventricular free wall was slightly increased in the untreated rats (0.21 ± 0.04 versus 0.19 ± 0.01 g in the controls; $P > 0.05$); this change was not improved by either treatment (0.22 ± 0.04 g with nitrendipine and 0.20 ± 0.03 g with benidipine; $P > 0.05$). Given that the LVDd assessed by echocardiography did not differ among the four experimental groups (Table 1), LV diastolic stiffness (LV end-diastolic pressure/LVDd) was significantly greater in the untreated group than in the controls (Fig. 3). Nitrendipine reduced this increase in stiffness to a level significantly lower than in the untreated group but still significantly higher than in the controls, whereas benidipine prevented it completely (Fig. 3).

Cardiomyocyte hypertrophy and interstitial fibrosis

Both the cardiomyocyte cross-sectional area (Fig. 4) and the level of interstitial fibrosis (Fig. 5) in the left ventricle were significantly greater in untreated rats than in control animals at 18 weeks of age. Benidipine or nitrendipine reduced the extents of both cardiomyocyte hypertrophy and interstitial fibrosis, but these parameters still remained significantly higher than the control values.

Coronary capillary density and diameter

Immunostaining revealed that the ratio of the number of coronary capillaries to that of cardiomyocytes was significantly greater in the untreated than in the control group; the ratio in the benidipine group was significantly greater than in the untreated and nitrendipine groups (Fig. 6a, b). Capillary density, however, was significantly lower in the untreated group than in the control group as a result of the cardiomyocyte hypertrophy evident in untreated animals (Figs 4a and 6c). Benidipine restored capillary density to the level in the control group, despite the residual cardiomyocyte hypertrophy in benidipine-treated rats. As indicated in the representative sections stained for CD31 (Fig. 6a), more coronary capillaries appeared to be dilated in untreated or nitrendipine-treated rats than in control or benidipine-treated animals. Indeed, quantitative analysis revealed that the diameter of coronary

capillaries was significantly smaller in benidipine-treated rats than in animals of the other three experimental groups (Fig. 6d).

Gene expression

Hemodynamic overload resulted in up-regulation of the expression of the genes encoding β -MHC, ACE, TGF- β 1, and collagen types I and III in the left ventricles of untreated rats at 18 weeks of age (Table 2). It also increased the ratio of the amount of collagen type I mRNA to that of collagen type III mRNA, an indicator of LV stiffness [23,25]. These increases in gene expression, with the exception of the increase in collagen type III mRNA abundance, were partly inhibited by treatment with benidipine or nitrendipine. Expression of the gene encoding the α 1G subunit of T-type Ca^{2+} channels was also significantly higher in the untreated group than in the controls; this increase was reduced to a greater extent by treatment with benidipine than by that with nitrendipine (Table 2). The levels of VEGF, HIF-1 α , eNOS, and Flt-1 mRNAs were significantly lower in the left ventricles of untreated-group rats than in the control-group rats; these changes were improved by treatment with benidipine but not with nitrendipine (Figs 7a–c and 8b). Similarly, benidipine, but not nitrendipine, significantly increased the levels of VEGF, HIF-1 α , and p-eNOS proteins compared with those in the untreated group (Fig. 7d–f). However, there were no differences in the levels of eNOS protein among the four experimental groups (Fig. 8a).

Renal fibrosis

Histological analysis showed that the level of interstitial fibrosis was significantly greater in the kidneys of untreated rats than in control animals at 18 weeks of age; this change was improved by benidipine or nitrendipine, but these parameters remained significantly higher than the control values (Fig. 8c and d). The level of serum creatinine was higher in untreated rats than in control rats (0.41 ± 0.04 versus 0.28 ± 0.3 mg/dl, respectively; $P < 0.05$); this change was not improved by either treatments (0.41 ± 0.10 mg/dl with benidipine and 0.45 ± 0.09 mg/dl with nitrendipine; $P > 0.05$).

Discussion

We showed that benidipine, but not nitrendipine, promoted coronary angiogenesis, likely accounting for the reduction in both LV diastolic stiffness and death rate induced by treatment with this drug in our animal model of hypertensive DHF.

Effects of benidipine on diastolic dysfunction and mortality

We inspected the general condition of Dahl salt-sensitive rats in each experimental group every day, and we found that high-salt diet rats developed rapid and labored respiration. All animals that died were immediately subjected to postmortem examination, including macroscopic inspection of the intracranial, thoracic, and abdominal cavities. Cerebral hemorrhage or infarction, aortic rupture, or colonic ischemia was not detected, but all animals that died before 18 weeks of age manifested marked pulmonary congestion (as evidenced by an increase in the ratio of lung weight to body weight), indicating that congestive heart failure – not stroke – was the major cause of death.

The diastolic dysfunction that develops in this animal model has been well characterized [5]. The time constant of LV pressure decay increases in association with the development of compensatory LVH. This time constant does not increase further with the development of DHF, which is accompanied instead by an increase in LV end-diastolic pressure. The relaxation delay is thus not likely to be a major contributor to the increase in end-diastolic pressure [26]. Our untreated group exhibited significant increases in both the time constant of LV pressure decay and LV end-diastolic pressure. Although benidipine and nitrendipine

improved relaxation properties to similar extents, benidipine induced a greater reduction in the acute pressure increase from the end of relaxation to late diastole (Fig. 3a), resulting in prevention of DHF and improved survival.

Effect of benidipine on left-ventricular diastolic stiffness

Excess interstitial fibrosis or collagen deposition is associated with increased diastolic stiffness and pulmonary edema or congestive heart failure [27,28]. Among various mechanisms that contribute to LV diastolic stiffness [26], abnormalities in the transcriptional or post-transcriptional regulation of collagen genes result in the disproportionate accumulation of fibrous tissue during the development of LVH. Increased LV diastolic stiffness during the development of DHF has been associated with increased interstitial fibrosis in hypertensive Dahl salt-sensitive rats [2,5]. However, although we found that benidipine reduced LV diastolic stiffness to a greater extent than did nitrendipine, the inhibitory effects of the two drugs on both interstitial fibrosis and the expression of profibrotic genes were similar. These results thus suggested that the superior effect of benidipine on LV diastolic stiffness was attributable to an action other than inhibition of interstitial fibrosis.

Effect of benidipine on coronary angiogenesis

In this study, we observed that the capillary-to-cardiomyocyte ratio was increased by 14%, and the capillary density was decreased by 18%, in the left ventricles of Dahl salt-sensitive rats in the untreated group, compared with the corresponding values in control animals. Benidipine induced a further increase in the capillary-to-cardiomyocyte ratio (40% increase compared with the control group), resulting in complete restoration of capillary density; nitrendipine had no such effects. Coronary capillaries were significantly smaller in the benidipine group than in the other three groups; most of the capillaries in the untreated and nitrendipine groups appeared dilated. These observations suggest that the decreased capillary ratio and density present in the untreated and nitrendipine groups may dilate fully to maximize blood flow, whereas the coronary reserve is sufficient in the benidipine group. Alternatively, the large capillaries observed in the untreated and nitrendipine groups may be mature, whereas the small vessels in the benidipine group may be newly generated. Given that angiogenesis results in degradation of surrounding interstitial fibrosis, benidipine might have been expected to reduce interstitial fibrosis to a greater extent than did nitrendipine; however, the two drugs had similar effects on cardiac fibrosis.

Effects of benidipine on vascular endothelial growth factor, hypoxia-inducible factor-1 α , and endothelial nitric oxide synthase expression

Cardiomyocyte hypertrophy is thought to increase diffusion distance, contributing to a reduction in oxygen supply to the myocardium. Neovascularization associated with cardiac hypertrophy may be attributable to up-regulation of the expression of angiogenic factors in cardiomyocytes. HIF-1 α is a transcription factor that induces expression of the VEGF gene in response to hypoxia–ischemia [29]. Angiogenesis in the vicinity of hypertrophic cardiomyocytes played an important role in preventing the transition from cardiac hypertrophy to LV systolic dysfunction in a mouse model of LVH [30]. In our model, coronary angiogenesis associated with cardiomyocyte hypertrophy was also implicated in preventing the transition from cardiac hypertrophy to DHF. When cardiac hypertrophy reaches a certain extent, even if ischemia persists, HIF-1 α synthesis is down-regulated, with the result that the production of VEGF and angiogenesis also cease [30]. The VEGF-induced activation of protein kinase AKT and consequent phosphorylation of eNOS play a central role in angiogenesis [31,32]. Up-regulation of eNOS has also been found to modify angiogenesis in ischemic tissues [33]. It has previously been reported that the drug had relatively selective blocking action on T-type Ca²⁺ channel, and favorable actions were

shown in cardiovascular system [10,19]. Here, we have demonstrated that benidipine (but not nitrendipine) increased the levels of HIF-1 α , VEGF, and eNOS mRNAs and proteins in the LV tissues of DHF rats. Taken together, these findings suggested that the blockade of cardiac T-type Ca²⁺ channel promotes ischemia-induced angiogenic response by enhancing HIF-1 α -mediated VEGF and eNOS expressions in the myocardium of DHF rats. It should be noted that there was a discrepancy in cardiac T-type Ca²⁺ channel blockade-mediated angiogenic action associated with HIF-1 α /eNOS/VEGF signaling pathway activation and morphological and functional improvement with the two drug interventions. Dihydropyridine nitrendipine has been shown to inhibit mineralocorticoid receptor activation *in vitro* and/or *in vivo* [34]. Recent studies have demonstrated that mineralocorticoid receptor blockade by antagonists results in attenuation of LV hypertrophy and heart failure in humans and in the Dahl salt-sensitive rat model [35,36]. Taken together, these findings suggest that the nitrendipine-mediated improvements in LV hypertrophy, fibrosis, and function are not attributable to the angiogenic action-linked HIF-1 α /eNOS/VEGF signaling pathway activation by T-type Ca²⁺ channel blockade, but rather to the inhibition of mineralocorticoid receptor activation.

Relationship between cardiomyocyte hypertrophy-associated angiogenesis and left-ventricular diastolic stiffness

A mismatch between the number of capillaries and the size of cardiomyocytes, resulting in myocardial hypoxia, is thought to arise during the development of cardiac hypertrophy [37,38]. A relationship between cardiac angiogenesis, cardiac hypertrophy, and cardiac systolic function is also thought to exist [39–41]. With regard to diastolic function, the hypertrophic myocardium appears especially susceptible to nitric oxide donors; this resulted in a marked reduction in LV end-diastolic pressure in one clinical study [42]. Our present results suggest that benidipine reduces diastolic stiffness and prevents the transition from compensatory LVH to DHF, not only by inhibiting the development of interstitial fibrosis but also by promoting coronary angiogenesis. Improved blood flow and function are associated with evidence of angiogenesis in an ischemic region of heart [43]. Because benidipine promoted coronary angiogenesis and improved LV diastolic stiffness, it is plausible that benidipine increases myocardial blood flow and myocardial function in a genomic and/or nongenomic way. Hypoxia increases isovolumic resting tension in the isolated guinea pig heart [44] and raises the diastolic PV curve in humans during balloon coronary angioplasty [45]. Further studies to clarify the influence of ischemia on cardiomyocyte resting tension or cardiomyocyte distensibility in hypertensive DHF are warranted.

Study limitations

Up-regulation of T-type Ca²⁺ channels has been associated with both hypertensive LVH [21] and DHF [12]. We compared the effects of benidipine, a blocker of T-type and L-type Ca²⁺ channels, with those of nitrendipine, a blocker of L-type Ca²⁺ channel [22], in a rat model of hypertensive DHF. Inhibition of T-type Ca²⁺ channels by benidipine may underlie the promotion of angiogenesis by this drug. Current through these channels and expression of their α 1G subunit are increased in association with the development of LVH [12,37]. Here, we could not measure T-type Ca²⁺ channel current in isolated cardiomyocytes from rats in the four experimental groups. Whereas benidipine and nitrendipine each inhibited the progression of LVH to similar extents, benidipine inhibited the increase in abundance of the α 1G subunit mRNA apparent in the left ventricle of untreated Dahl salt-sensitive rats to a greater extent than did nitrendipine, suggesting that current through these channels was reduced by benidipine treatment. Further studies are warranted to clarify the relationship between inhibition of T-type Ca²⁺ channel current and coronary angiogenesis during the development of DHF. In addition, it is better to calculate peak flow velocities at the mitral

level during rapid filling (*E*) and during atrial contraction (*A*), as well as the *E/A* ratio, the deceleration time, and the isovolumic relaxation time, from the pulsed Doppler echocardiographic data for assessment of LV diastolic function. Our study did not include an evaluation of these cardiac diastolic function indices.

Clinical implications

We have demonstrated that benidipine reduced LV diastolic stiffness and increased survival in hypertensive Dahl salt-sensitive rats to a greater extent than did nitrendipine. The prevention of DHF by benidipine appeared to be due predominantly to the promotion of angiogenesis rather than to inhibition of interstitial fibrosis, and this effect on angiogenesis appeared to be mediated by up-regulation of the production of HIF-1 α , VEGF, and eNOS. Benidipine may thus be more effective than purely L-type Ca²⁺ channel blockers in preventing hypertensive DHF. Given that ARBs and ACEIs are widely used and are more effective than CCBs for the treatment of LVH, the latter drugs are considered to be potential second-line agents in combination therapy. The effects of combinations of ARBs or ACEIs with different types of CCBs, including a blocker of T-type and L-type Ca²⁺ channels benidipine, thus warrant testing in clinical trials.

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Abbreviations

β-MHC	β -myosin heavy chain
ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitor
ARB	angiotensin-receptor blocker
DHF	diastolic heart failure
DHP	dihydropyridine
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF-1α	hypoxia-inducible factor-1 α
IVST	interventricular septum
LV $dP/dt_{\max}$	maximum first derivative of left ventricular pressure
LV $dP/dt_{\min}$	minimal rate of left ventricular pressure
LVDd	left-ventricular end-diastolic dimension
LVDs	left-ventricular end-systolic dimension
LVH	left-ventricular hypertrophy
LV	left-ventricular
MR	mineralocorticoid receptor

PCR	polymerase chain reaction
$T_{1/2}$	the pressure half-time
TGF-1β	transforming growth factor-1 β
VEGF	vascular endothelial growth factor

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Fig. 1.

Time course of systolic blood pressure in Dahl salt-sensitive rats fed a high-salt diet from 7 weeks of age and treated with vehicle (untreated group), benidipine (3 mg/kg daily), or nitrendipine (10 mg/kg daily) from 10 weeks of age, as well as in age-matched controls fed a low-salt diet (control group). Data are means $\pm$ SEM ($n = 10$ per group).

Fig. 2. Kaplan-Meier plots of survival rates of Dahl salt-sensitive rats in the four experimental groups ($n = 10$ per group).

Fig. 3. Left-ventricular (LV) pressure recordings and LV diastolic stiffness values in Dahl salt-sensitive rats of the four experimental groups at 18 weeks of age. (a) Representative waveforms obtained from a pressure manometer inserted into the left ventricle. (b) LV diastolic stiffness (LV end-diastolic pressure/LV end-diastolic dimension). Data are means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ versus control group; † $P < 0.05$ versus untreated group; ‡ $P < 0.05$ versus nitrendipine group.

Fig. 4. Cardiomyocyte hypertrophy in Dahl salt-sensitive rats of the four experimental groups at 18 weeks of age. (a) Representative hematoxylin–eosin staining of sections of the left ventricle. Scale bar, 100 μm. (b) Cross-sectional cardiomyocyte area, as measured in 10 randomly chosen microscopic fields from three different sections in each tissue block, similar to those shown in (a). Data are means ± SEM ($n = 5$ per group). * $P < 0.05$ versus control group; † $P < 0.05$ versus untreated group.

Fig. 5. Interstitial fibrosis in the left ventricle of Dahl salt-sensitive rats in the four experimental groups at 18 weeks of age. (a) Representative Azan-Mallory staining of sections of the left ventricle. Scale bar, 200 μm. (b) Percentage area of interstitial fibrosis measured in 10 randomly chosen microscopic fields from three different sections in each tissue block, similar to those shown in (a). Data are means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ versus control group; † $P < 0.05$ versus untreated group.

Fig. 6. Coronary capillaries in the left ventricle of Dahl salt-sensitive rats in the four experimental groups at 18 weeks of age. (a) Representative CD31 immunostaining of sections of the left ventricle. Scale bar, 100 μm . (b–d) Ratio of the number of coronary capillaries to that of cardiomyocytes (b); capillary density (c); and capillary diameter (d) were measured in 15 randomly chosen microscopic fields from three different sections in each tissue block, similar to those shown in (a). The number of capillaries was measured in 15 randomly chosen microscopic fields from three different sections in each tissue block. Quantitative data are means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ versus control group; † $P < 0.05$ versus untreated group; ‡ $P < 0.05$ versus nitrendipine group.

Fig. 7.

Expression of VEGF, HIF-1, and eNOS in the left ventricle of Dahl salt-sensitive rats in the four experimental groups at 18 weeks of age. (a–c) Abundances of VEGF, HIF-1, and eNOS mRNAs, respectively, as determined by RT and real-time PCR analysis. Data were normalized by the amount of GAPDH mRNA and then expressed relative to the normalized value for the control group; they are means $\pm$ SEM ($n = 5$ per group). (d–f) Abundances of VEGF, HIF-1, and eNOS proteins, respectively, as determined by immunoblot analysis. Representative blots are shown in the upper panels, and quantitative data (means $\pm$ SEM) are presented in the lower panels. The amount of each protein was normalized by that of GAPDH and then expressed relative to the normalized value for the control group. * $P < 0.05$ versus control group; $^{\dagger}P < 0.05$ versus untreated group; $^{\ddagger}P < 0.05$ versus nitrendipine group ($n = 5$ per group). eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor.

Fig. 8.

Levels of total eNOS (t-eNOS) protein, Flt-1 mRNA, and renal interstitial fibrosis in the four experimental groups at 18 weeks of age. (a) Abundance of t-eNOS protein, as determined by immunoblot analysis. (b) Abundance of Flt-1 mRNA, as determined by real-time PCR analysis. (c) Representative Azan-Mallory stainings of sections of the left ventricle. Scale bar, 100 μm. (d) Percentage area of interstitial fibrosis measured in 10 randomly chosen microscopic fields from three different sections in each tissue block, similar to those shown in (c). Data are means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ versus control group; † $P < 0.05$ versus untreated group; ‡ $P < 0.05$ versus nitrendipine group. eNOS, endothelial nitric oxide synthase.

Table 1

Hemodynamic, echocardiographic, and pathological parameters in Dahl salt-sensitive rats of the four experimental groups at 18 weeks of age

Parameter	Control	Untreated	Benidipine	Nitrendipine
SBP (mmHg)	172 ± 3	253 ± 5 [*]	234 ± 9 ^{*,**}	235 ± 7 ^{*,**}
Heart rate (b.p.m.)	466 ± 18	524 ± 12	450 ± 13 ^{*,**,*}	511 ± 7
IVST (mm)	1.91 ± 0.05	2.50 ± 0.12 [*]	2.10 ± 0.06 ^{*,**}	2.20 ± 0.08 ^{*,**}
LVDd (mm)	8.07 ± 0.16	7.81 ± 0.11	7.97 ± 0.33	8.00 ± 0.40
LVFS (%)	44.7 ± 1.5	42.5 ± 3.6	44.0 ± 2.8	44.2 ± 3.8
LV $dP/dt_{\max}$ (mmHg/s)	8271 ± 260	7890 ± 156 [*]	10234 ± 347 ^{*,**}	10877 ± 932 ^{*,**}
LV $dP/dt_{\min}$ (mmHg/s)	7654 ± 324	7022 ± 108 [*]	7634 ± 434 ^{*,**}	8023 ± 663 ^{*,**}
$T_{1/2}$ (ms)	3.8 ± 0.2	6.2 ± 0.3 [*]	4.9 ± 0.3 ^{*,**}	5.1 ± 0.3 ^{*,**}
LVEDP (mmHg)	3.9 ± 0.2	11.7 ± 0.2 [*]	3.8 ± 0.4 ^{*,**,*}	8.1 ± 0.3 ^{*,**}
LV weight (mg)/BW (g)	2.11 ± 0.02	4.20 ± 0.12 [*]	2.94 ± 0.07 ^{*,**}	3.05 ± 0.16 ^{*,**}
Lung weight (mg)/BW (g)	2.76 ± 0.07	8.01 ± 1.01 [*]	3.51 ± 0.12 ^{*,**,*}	6.11 ± 1.06 ^{*,**}

SBP, systolic blood pressure; IVST, interventricular septum thickness; LVDd, left-ventricular end-diastolic dimension; LVFS, left-ventricular fractional shortening; LV $dP/dt_{\max}$ and LV $dP/dt_{\min}$, first derivative of maximum or minimum left ventricular pressure, respectively, with respect to time; $T_{1/2}$, time constant of LV pressure decay; LVEDP, left-ventricular end-diastolic pressure; LV weight, weight of the left ventricle; BW, body weight. Data are means ± SEM ($n = 5$ per group).

* $P < 0.05$ versus control group.

** $P < 0.05$ versus untreated group.

*** $P < 0.05$ versus nitrendipine group.

Table 2

Expression of prohypertrophic, profibrotic, or proinflammatory genes in the left ventricles of Dahl salt-sensitive rats in the four experimental groups at 18 weeks of age

Gene	Control	Untreated	Benidipine	Nitrendipine
β-MHC	1.0 ± 0.2	3.7 ± 0.7 *	1.9 ± 0.2 *,**	1.9 ± 0.2 *,**
TGF-β1	1.0 ± 0.1	2.0 ± 0.3 *	1.2 ± 0.1 **	1.2 ± 0.2 **
ACE	1.0 ± 0.2	1.7 ± 0.1 *	1.1 ± 0.1 **	1.0 ± 0.1 **
Collagen type I	1.0 ± 0.2	4.0 ± 0.7 *	2.6 ± 0.4 *,**	2.5 ± 0.3 *,**
Collagen type III	1.0 ± 0.2	3.0 ± 0.5 *	2.6 ± 0.3 *	2.5 ± 0.2 *
Collagen type I/III ratio	1.0 ± 0.1	1.4 ± 0.2 *	1.1 ± 0.1 **	1.1 ± 0.1 **
TCC α1G	1.0 ± 0.1	3.2 ± 0.6 *	1.2 ± 0.2 *,**,***	2.4 ± 0.3 *,**

The amount of each mRNA in the left ventricle was determined by RT and real-time PCR analysis, normalized by that of GAPDH mRNA, and expressed relative to the normalized value for the control group. TCC α1G, α1G subunit of T-type Ca²⁺ channels. Data are means ± SEM (*n* = 5 per group).

* *P* < 0.05 versus control group.

** *P* < 0.05 versus untreated group.

*** *P* < 0.05 versus nitrendipine group.