Benidipine, a long-acting calcium channel blocker, inhibits cardiac remodeling in pressure-overloaded mice

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Objective: The effects of long-acting calcium channel blockers (CCBs) on pressure overload-induced cardiac remodeling are seldom studied in animals. We evaluated the effects of benidipine, a long-acting CCB, on cardiac remodeling.

Methods: Rat neonatal cardiac myocytes were used to examine the influence of benidipine on protein synthesis. Cardiac remodeling was induced in C57 B6/J mice by transverse aortic constriction (TAC). Then the effects of benidipine (10 mg/kg/d) were assessed on myocardial hypertrophy and heart failure, cardiac histology, and gene expression.

Results: Benidipine significantly inhibited protein synthesis by cardiac myocytes stimulated with phenylephrine (PE), and this effect was partially abolished by cotreatment with a nitric oxide synthase (NOS) inhibitor [N(G)-nitro-l-arginine methylester (l-NAME)]. Four weeks after the onset of pressure overload, benidipine therapy potently inhibited cardiac hypertrophy and prevented heart failure. The heart to body weight ratio was 6.89 ± 0.48 mg/g in treated mice vs. 8.76 ± 0.33 mg/g in untreated mice (<i>P</i> < 0.01), and the lung to body weight ratio was 7.39 ± 0.93 mg/g vs. 10.53 ± 0.99 mg/g, respectively (P < 0.05). Left ventricular fractional shortening (LVFS) was improved on echocardiography. Plasma NO levels were increased, while B type natriuretic peptide, protein inhibitor of neuronal NOS, and procollagen IV alpha were down-regulated in benidipine-treated mice.

Conclusion: These results indicate that benidipine inhibits cardiac remodeling due to pressure overload at least partly by acting on the nitric oxide signaling pathway.

Keywords: Calcium channel blocker; Heart failure; Hypertrophy; Gene expression

1. Introduction

Calcium channel blockers (CCBs) are one of the most frequently used classes of drugs for the treatment of hypertension. Although early clinical studies showed a disappointing outcome when short-acting dihydropyridine CCBs were used to reduce cardiovascular risk [1,2], well-designed prospective randomized controlled clinical trials have demonstrated that long-acting dihydropyridine CCBs are effective for reduction of the blood pressure (BP), inhibition of cardiac remodeling, and decreasing the risk of cardiovascular endpoints [3]. However, the underlying mechanism of the beneficial effect of CCBs on cardiac remodeling is not fully understood. An earlier study performed by our laboratory showed that the vasodilator hydralazine significantly lowered the systemic blood pressure but did not exert any effect on cardiac hypertrophy induced in rats by N(G)-nitro-l-arginine methylester (l-NAME), a nitric oxide (NO) synthase inhibitor [4], suggesting that blood pressure reduction alone was not sufficient to inhibit cardiac remodeling. We also
reported that a long-acting CCB, benidipine, could increase coronary flow and reduce myocardial ischemia by promoting the release of NO [5,6]. NO is also known to lessen the severity of cardiac hypertrophy and heart failure [7,8]. Furthermore, benidipine has been demonstrated to inhibit myocardial fibrosis in diabetic rats [9]. Based on these lines of evidence, we hypothesized that benidipine may inhibit cardiac remodeling via the NO signaling pathway.

Because the occurrence of cardiac remodeling has been shown to be associated with subsequent cardiovascular events, therapeutic approaches that inhibit cardiac remodeling are likely to improve the prognosis. Chronic left ventricular pressure overload induced by transverse aortic constriction (TAC) is a well established animal model for investigation of cardiac remodeling [10–12], but few experimental studies have attempted to clarify the effects of long-acting CCBs on cardiac remodeling using this model. Therefore, we evaluated the effects of benidipine on cardiac hypertrophy and heart failure in a murine model of pressure overload due to TAC and explored the mechanisms involved.

2. Methods

2.1. Cell culture

Rat neonatal cardiac myocytes were isolated, as described previously [13]. The myocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% FBS (Equitech-Bio), which was changed to serum-free medium after 72 h. Cells were cultured under serum-free conditions for 48 h before agents were added. Protein synthesis by cultured cells was evaluated from [3H] leucine incorporation, as described elsewhere [11,13]. Cardiac myocytes were exposed to 10−4 M phenylephrine (PE) for 24 h in the presence or absence of benidipine (kindly provided by the Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo Sunto, Shizuoka, Japan), and the increase of [3H] leucine uptake was examined. To determine whether the NO signaling pathway was involved in the inhibition of protein synthesis by cardiac myocytes, we examined whether the in vitro effect of benidipine could be blocked by the NO synthase (NOS) inhibitor L-NAME (10−5 M).

2.2. Animal model

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Male C57BL/6J mice aged 8–9 weeks and weighing 19–23 g were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) injected intraperitoneally. Then pressure overload was created, as described previously [10]. Briefly, endotracheal intubation was performed, and the cannula was connected to a volume-cycled rodent ventilator with a tidal volume of 0.5 ml (room air) and a respiration rate of 100/min. The chest was entered via the second intercostal space at the upper left sternal border. After the arch of the aorta was isolated, TAC was created using a 7–0 suture tied twice around a 27-gauge needle and the aortic arch between the innominate and left common carotid arteries. After the suture was tied, the needle was gently removed, yielding 60–80% constriction of the aorta. More than 1000 murine TAC models have been created at our laboratory, and cardiac hypertrophy occurs in 100% of these animals. The dispersion of heart weight to body weight ratio evaluated with statistical parameter coefficient variance at 4 weeks following TAC is about 20%, as we reported previously [11,14].

To test whether benidipine could inhibit the cardiac hypertrophy due to TAC, we treated the mice with either saline (TAC group) or benidipine at 10 mg/kg/d (po, mixed with 0.3% carboxymethyl cellulose sodium and suspended in water) from the 2nd day after surgery. The benidipine dose was based on previous reports from our [4] and another [9] laboratory as well as a preliminary study. To confirm that the extent of the pressure overload was similar between benidipine-treated and untreated animals, three mice were randomly selected from each group to measure the pressure in ascending aorta, using a 1.4 F Millar Pressure Catheter on the 2nd day after TAC. Four weeks after the creation of pressure overload, both the tail cuff blood pressure (BP) and the heart rate (HR; BP-98A, Softron, Tokyo, Japan) were measured 1 day before sacrifice. LV hemodynamic studies were performed by cannulation of the right carotid artery with a Millar Pressure Catheter that was carefully advanced to the LV. Then the mice were killed to measure organ weights and to perform histological analysis.

2.3. Histological examination

The cross-sectional area of cardiac myocytes and the extent of myocardial fibrosis were measured, as described elsewhere [4,15]. Briefly, the cardiac myocyte area and myocardial fibrosis area were analyzed quantitatively by morphometry of either HE-stained or Azan/Mallory-stained sections. The original images were digitized and transformed into binary images, after which the cardiac myocyte area or fibrosis area was calculated with an automatic area quantification program (NIH Image). One hundred myocytes per heart were counted, and the average value was determined. The total myocardial fibrosis index was defined as the sum of the total area of fibrosis in the entire microscopic field divided by the sum of total connective tissue area plus the myocardial area in the entire field.

2.4. Echocardiography

Transathoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHZ transducer (Philips, the Netherlands). Mice were weighed, lightly anesthetized with
2.5% avertin (0.06 ml/10 g), and set in the left lateral
decubitus position or the supine position. After the mouse
recovered to complete consciousness (about 10 min), two-
dimensional short-axis views of the left ventricle were
obtained for guided M-mode measurement of the left
ventricular diastolic posterior wall thickness (LVPWd), left
ventricular end-diastolic dimension (LVEDd), and left
ventricular end-systolic dimension (LVEsd). Left ventricu-
lar fractional shortening (LVFS) was calculated as follows:
LVFS=(LVEDd−LVEsd)/LVEDd*100.

2.5. Microarray analysis

To determine the gene expression profile during cardiac
remodeling, we performed microarray studies of murine
hearts after pressure overload for 1 or 4 weeks. Data about
the time course of the induction of NO synthase and fibrosis-
related genes were needed to investigate their roles in cardiac
hypertrophy and heart failure. Total RNA was prepared from
murine hearts using Triazol (Gibco-BRL), according to the
manufacturer’s instructions. Microarray hybridization was
performed in duplicate using Affymetrix Murine Genome
U74v2A gene chips and RNA from hearts of animals in the
TAC or sham operation groups at 1 or 4 weeks after surgery.
Data were analyzed using Genespring 6 software [16].

2.6. Measurement of plasma nitric oxide

Blood was obtained from the right ventricle with a 23-
gauge needle at the time of sacrificing the mice. The plasma
concentrations of NOx (NO2+NO3) was measured with an
autoanalyzer (ENO-10, Eicom Kyoto, Japan), as described
elsewhere [5,6,17]. Samples were applied to an analytical
column that was connected to a copperized cadmium
reduction column to reduce NO2 to NO3, which was then
reacted with Griess reagent, and the absorbance of the
product was measured at 540 nm.

2.7. Quantitative PCR

Based on the results of microarray analysis, we chose
three genes that were consistently up-regulated at both 1 and
4 weeks after the onset of LV pressure overload and were
closely related to cardiac hypertrophy or heart failure. We
further investigated the effects of benidipine on these genes
by real-time PCR. The three genes were the natriuretic
peptide precursor type B (BNP) gene, protein inhibitor of
neuronal nitric oxide synthase (PIN) gene, and procollagen
IV alpha gene. Primers were designed using Gene Express
software. Using 50 ng/ml of total RNA as the template,
quantitative measurement was performed with an ABI Prism
7700 sequencing system. Amplification was done by the
one-step method using a Quantitect SYBR Green RT-PCR
kit (QIAGEN). Glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) was amplified as an endogenous control, and
quantitation of target gene levels was performed relative to
this gene.

2.8. Statistical analysis

For all statistical tests, multiple comparison was per-
formed by one-way ANOVA with the Tukey–Kramer exact
probability test. The least-squares method was used for
linear correlation between selected variables. Results are
reported as the mean±S.E.M., and P<0.05 was considered
statistically significant.

3. Results

3.1. Benidipine reduces cardiac myocyte protein synthesis
stimulated by PE

Benidipine (10^{-4} M) did not affect basal [3H] leucine
uptake by cardiac myocytes, but it inhibited PE-induced
protein synthesis in a concentration-dependent fashion (Fig. 1A). The enlargement of cells induced by PE was also inhibited by benidipine (Fig. 1B). The inhibitory effect of benidipine on PE-induced protein synthesis was partially blocked by L-NAME (Fig. 1C).

3.2. Benidipine inhibits pathological cardiac hypertrophy

The hemodynamic and echocardiographic data obtained just before sacrifice are shown in Table 1. Benidipine (10 mg/kg/d) did not significantly affect the tail cuff systolic blood pressure, but the LV wall was thinner, and LV dimensions were smaller in benidipine-treated mice than in TAC mice (Table 1).

Echocardiography and hemodynamics showed no differences among the three groups of mice before surgery (data not shown). The ascending aortic systolic blood pressure was measured on the 2nd day after TAC or sham operation without drug treatment in order to evaluate the extent of pressure overload (in three mice per group), no significant difference was noted between the TAC and benidipine groups (98±5 mm Hg in the sham group, 163±4 mm Hg in the TAC group, and 161±3 mm Hg in the benidipine group).

LV hemodynamics were similar between TAC mice with or without benidipine treatment (Fig. 2), suggesting that an oral dose of 10 mg/kg did not significantly affect LV function.

Consistent with the in vitro results, benidipine markedly inhibited cardiac hypertrophy at 4 weeks after TAC (Fig. 3). Histological examination showed that the extent of myocyte hypertrophy (Fig. 4A,B) was reduced and that myocardial fibrosis was less severe in benidipine-treated mice (Fig. 4C,D).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>HR (bpm)</th>
<th>SBP (mm Hg)</th>
<th>LVPWd (mm)</th>
<th>LVEDd (mm)</th>
<th>LVESd (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>25.2±0.4**</td>
<td>651±11</td>
<td>114±3</td>
<td>0.65±0.02***</td>
<td>3.07±0.06</td>
<td>1.64±0.04**</td>
</tr>
<tr>
<td>TAC</td>
<td>22.64±0.41</td>
<td>686±26</td>
<td>101±5</td>
<td>0.98±0.04</td>
<td>3.38±0.12</td>
<td>2.29±0.04</td>
</tr>
<tr>
<td>TAC+Beni</td>
<td>23.1±0.4</td>
<td>652±26</td>
<td>105±2</td>
<td>0.77±0.03***</td>
<td>3.04±0.06*</td>
<td>1.69±0.12**</td>
</tr>
</tbody>
</table>

Beni—benidipine (10 mg/kg/d po); BW—body weight; HR—heart rate; SBP—Tail cuff systolic blood pressure; LVPWd—LV diastolic posterior wall thickness; LVEDd—LV end-diastolic dimension; LVESd—left ventricular end-systolic dimension. The number of mice in the sham, TAC, and TAC+benidipine groups was 10, 17, and 11, respectively, for BW, LVPWd, LVEDd, and LVESd; and 10, 9, and 7 for HR and SBP.

* P<0.05.
** P<0.01.
*** P<0.001 vs. TAC (transverse aortic constriction).

Fig. 2. Left ventricular (LV) hemodynamics measured with a Millar Catheter at 4 weeks after TAC. (A) LV pressure and dp/dt in the TAC and benidipine groups. (B) No significant differences of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were noted between TAC mice with or without benidipine. (C) Dp/dt max was closely correlated with LVSP in untreated mice. (D) Dp/dt max/LVSP was not significantly increased in benidipine-treated mice.
3.3. Benidipine prevents progression from hypertrophy to heart failure

TAC induced congestive heart failure with a reduction in LVFS and increase of pulmonary congestion. LVFS measured by echocardiography was significantly higher in benidipine-treated mice than in TAC mice (Fig. 5A,B). Compared with the value for sham-operated mice, the lung weight to body weight ratio (LW/BW) was increased by about 108% in TAC mice, but only rose by 46% in benidipine-treated mice (Fig. 5C,D).

![Fig. 3. Benidipine inhibits cardiac remodeling. (A) Representative pictures of whole hearts. (B) The heart to body weight ratio (HW/BW) was significantly decreased in TAC mice treated with benidipine (10 mg/kg/d) compared with untreated TAC mice.](image)

![Fig. 4. Results of histological examination. (A) Representative images of the myocardium (HE stain ×200). (B) The cross-sectional area of cardiac myocytes was significantly increased in TAC mice by pressure overload for 4 weeks, while treatment with benidipine blunted the enlargement of myocytes. (C) Representative pictures of myocardial fibrosis (Azan–Mallory stain ×100). (D) Quantitative analysis showed that benidipine significantly inhibited myocardial fibrosis due to pressure overload for 4 weeks. Three hearts per group were used to determine the cross-sectional area of cardiac myocytes and the extent of myocardial fibrosis.](image)
3.4. BNP, PIN, and procollagen IV are up-regulated in cardiac hypertrophy

Based on evidence from our laboratory and other investigators that BNP is an important molecular marker of cardiac hypertrophy or heart failure, and that both NO and fibrosis play an important role in cardiac remodeling, we assessed the expression of the BNP, PIN, and procollagen IV alpha genes in pressure-overloaded murine hearts, using microarray analysis. We found that a series of hypertrophy-related genes were up-regulated (Fig. 6A), including the BNP, PIN, and procollagen IV alpha genes, which were consistently up-regulated at both 1 and 4 weeks after TAC. Expression of calmodulin and five other procollagen genes was also increased by pressure overload (Fig. 6B).

3.5. Benidipine increases plasma NOx and down-regulates BNP, PIN, and procollagen IV alpha

As shown in Fig. 7A, the plasma level of NOx was markedly decreased in TAC mice at 4 weeks and was significantly increased in TAC mice treated with benidipine. Quantitative RT-PCR (Fig. 7B–D) demonstrated that benidipine decreased the level of BNP, a molecular marker for hypertrophy, and also down-regulated the expression of PIN and procollagen IV alpha1. These changes supported our other findings in vitro and in vivo that benidipine inhibits cardiac hypertrophy and improves cardiac function partly by increasing the release of NO.

4. Discussion

4.1. Major findings

The present study is the first to evaluate the inhibitory effect of benidipine on cardiac remodeling induced by TAC in mice. The major findings of this study include the observations that (1) benidipine inhibits the increase of protein synthesis by cardiac myocyte stimulated by phenylephrine; (2) cardiac hypertrophy, myocardial fibrosis, and heart failure in pressure-overload mice were ameliorated by treatment with benidipine; and (3) an NO synthase inhibitor partially blocked the beneficial effect of benidipine on myocyte hypertrophy, while benidipine down-regulated protein inhibitor of neuronal nitric oxide synthase and increased the plasma NO level. These findings suggest that benidipine improves cardiac remodeling via an effect on the NO signaling pathway.
4.2. Role of NO in cardiac remodeling

NO has been recognized as an important regulator of cardiac remodeling since it can influence both cardiac hypertrophy and heart failure. NO has been reported to exert an antihypertrophic effect in the hearts of spontaneously hypertensive rats without changing the blood pressure\[18\], which is in agreement with the results of this study. It is generally recognized that hemodynamic factors regulate cardiac myocyte hypertrophy\[19\], but exceptions have also been frequently reported. We previously reported that hydralazine significantly reduces the systemic blood pressure but does not have any effect on cardiac hypertrophy. In contrast, some drugs inhibit cardiac myocyte hypertrophy in the absence of a significant effect on hemodynamic, as we have reported previously\[11,12,14\]. Exogenous NO has

Fig. 6. cDNA microarray analysis of pressure-overload or sham-operated murine hearts. (A) From a total of 12,488 genes, three target genes were selected. These genes were functionally related to cardiac hypertrophy, heart failure, and nitric oxide signaling or fibrosis. (B) The three target genes were significantly up-regulated at 1 and 4 weeks after TAC relative to the levels in corresponding sham mice. Calmodulin and five other procollagen genes also showed up-regulation in response to pressure overload. The number of mice tested in each group was two. *P<0.05 vs. sham at 1w, †P<0.05 vs. sham at 4W (ANOVA).

Fig. 7. Plasma nitric oxide level (A) and real-time PCR of the three target genes (B–D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. PIN—protein inhibitor of neuronal nitric oxide synthase; BNP—natriuretic peptide precursor type B. n=4 per group for real-time PCR.
also been demonstrated to cause dose-dependent inhibition of alpha1-adrenoceptor-stimulated protein synthesis in neonatal rat myocytes [7]. These results support our finding that benidipine caused a concentration-dependent decrease of PE (an alpha1-adrenoceptor agonist)-stimulated protein synthesis by cardiac myocytes, and that this effect was blunted by NO synthase inhibitor. In addition, benidipine attenuated cardiac hypertrophy in pressure-overload mice without a significant change of blood pressure, and this antihypertrophic effect was at least partially mediated via the down-regulation of myocardial PIN. PIN has been demonstrated to regulate three types of NO synthase (NOS) [20]. Since both neural NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed in the myocardium, consistent up-regulation of PIN during the progression of cardiac hypertrophy, as noted in this study, is likely to decrease the release of NO. Interestingly, our data showed that benidipine significantly increased circulating NO levels, providing direct evidence for the abovementioned hypothesis that NO may play an important role in regulating cardiac hypertrophy. Although we did not monitor the blood concentration of benidipine, the dose that we used was effective for increasing the production of NO and consequently for attenuating cardiac hypertrophy.

We also found that benidipine could ameliorate progression from cardiac hypertrophy to heart failure, as confirmed by echocardiography, assessment of pulmonary congestion, and measurement of BNP expression. These results are partially attributable to the increase in NO production. Indeed, we have previously reported that benidipine increases coronary blood flow and reduces the severity of myocardial ischemia via an NO-dependent mechanism [5], and benidipine also improves cardiac remodeling induced by the eNOS inhibitor L-NAME [4]. Studies using genetically engineered mice have provided substantial evidence for a critical role of NO in cardiac remodeling. After myocardial infarction, LV dilation is more marked, heart function is more severely impaired, and long-term mortality is higher in eNOS-deficient mice compared with wild-type mice [8]. In contrast, congestive heart failure is less severe, and survival is increased in eNOS transgenic mice receiving coronary ligation [21]. It is worth noting that the preventive effect of benidipine on progression to heart failure may be secondary to its antihypertrophic effect. Further studies are needed to examine whether benidipine is effective in animals or humans with chronic heart failure.

4.3. Fibrosis and cardiac remodeling

Fibrosis of the myocardium plays a pivotal role in the process of cardiac remodeling. In the present study, we found that benidipine could significantly inhibit myocardial fibrosis in pressure-overload mice, a result that agrees with previous findings [9]. Although collagen type I and collagen type III produced by cardiac fibroblasts are the major components of the myocardial collagen matrix, type IV collagen is also expressed by both cardiac myocytes and fibroblasts and is a major component of the basement membrane [22,23]. Type IV collagen was reported to be increased in the hearts of diabetic rat [24] and is found in the fibrotic cardiac lesions of patients with DCM [25]. The angiotensin II-induced increase of fibronectin mRNA in the myocardium is accompanied by a similar increase of type I collagen, type IV collagen, and atrial natriuretic factor steady-state mRNA [26]. In this study, cDNA microarray analysis showed significant up-regulation of procollagen IV alpha at both 1 and 4 weeks after TAC, suggesting that this may be a potentially important gene in cardiac remodeling. Down-regulation of this gene by benidipine might have made an important contribution to the inhibition of cardiac remodeling.

4.4. Benidipine and cardiac sympathetic activity

Long-term cardiac sympathetic activation is detrimental to the heart, so one of the major aims of antihypertensive therapy is to reduce sympathetic tone. Differences in the formulations and pharmacokinetics of CCBs have various clinical influences, altering the effect of these drugs on blood pressure, heart rate, and cardiac sympathetic activity. Short-acting dihydropyridine CCBs enhance noradrenaline release from the sympathetic nerves [27]. In contrast, evidence suggests that long-acting calcium antagonists do not significantly affect sympathetic tone and may exert a more favorable clinical effect [28–30]. Our data showed that benidipine did not increase the heart rate. Moreover, benidipine prevented progression from cardiac hypertrophy to failure, suggesting that it does not enhance sympathetic tone. It is even possible that benidipine counteracts sympathetic activation in cardiac hypertrophy by increasing the release of NO because a reduced action of NO often contributes to overall sympathetic excitation in heart failure (review [31]).

4.5. Perspectives

In summary, this study provided evidence of the beneficial effect of a long-acting calcium antagonist, benidipine, on cardiac remodeling. Benidipine inhibited cardiac myocyte hypertrophy both in vitro and in vivo and also inhibited progression from cardiac hypertrophy to failure due to LV pressure overload. These effects were potentially mediated via an influence on the NO signaling pathway.

The question of whether CCB therapy increases cardiovascular events has attracted worldwide attention. Recent clinical trials have largely settled this question [29,30,32], but CCBs are still linked with a slightly increased risk of heart failure. However, the PRAISE trial revealed that amlodipine, a long-acting CCB, was not associated with increased mortality or morbidity in patients with severe
CHF [29]. Our studies and other investigations have consistently confirmed that amlodipine increases NO production [4,10,33,34]. Benidipine may also be beneficial for patients with hypertension-induced CHF, but a well-designed clinical trial is needed to investigate this point.

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