Abstract—Chronic inhibition of nitric oxide (NO) synthesis induces cardiac remodeling independent of systemic hemodynamic changes in rats. We examined whether long-acting dihydropyridine calcium channel blockers block myocardial remodeling and whether the activation of 70-kDa S6 kinase (p70S6K) and extracellular signal-regulated kinase (ERK) are involved. Ten groups of Wistar-Kyoto rats underwent 8 weeks of drug treatment consisting of a combination of NO synthase inhibitor N^o-nitro-L-arginine methyl ester (L-NAME), an inactive isomer (D-NAME), amlodipine (1 or 3 mg/kg per day), or benidipine (3 or 10 mg/kg per day). In other groups, L-NAME was also used in combination with a p70S6K inhibitor (rapamycin), a MEK inhibitor (PD98059), and hydralazine. Systolic blood pressure (SBP), heart rate, and left ventricular weight (LVW) were measured, together with histological examinations and kinase assay. L-NAME increased SBP and LVW (1048 ± 22 versus 780 ± 18 mg, P < 0.01) compared with the control, showing a significant increase in cross-sectional area of cardiomyocytes after 8 weeks. Amlodipine, benidipine, or hydralazine equally attenuated the increase in SBP induced by L-NAME. However, both amlodipine and benidipine but not hydralazine attenuated the increase in LVW by L-NAME (789 ± 27, 825 ± 20 mg, P < 0.01, and 1118 ± 29 mg, NS, respectively), also confirmed by histological analysis. L-NAME caused a 2.2-fold/1.8-fold increase in p70S6K/ERK activity in myocardium compared with the control, both of which were attenuated by both amlodipine and benidipine but not hydralazine. Both rapamycin and PD98059 attenuated cardiac hypertrophy in this model. Thus, long-acting dihydropyridine calcium channel blockers inhibited cardiac hypertrophy induced by chronic inhibition of NO synthesis by inhibiting both p70S6K and ERK in vivo. (Hypertension. 2003;41:963-967.)

Key Words: hypertrophy □ nitric oxide □ L-NAME □ calcium channel blockers □ kinase

The long-acting dihydropyridine calcium channel blockers (CCBs), which are widely used in the clinical setting, have been shown to prevent cardiac remodeling, indicated by some clinical and experimental reports.1,2 However, the subcellular signaling mechanisms of this cardioprotective effect are not well understood. To clarify these issues, we used the in vivo Wistar-Kyoto rat model with chronic inhibition of nitric oxide (NO) synthesis by N^o-nitro-L-arginine methyl ester (L-NAME). NO is known to mediate vasodilation, inhibit platelet aggregation, and prevent leukocyte adherence to endothelial cells.3 The critical feature of this rat model lacking NO synthesis is persistent activation of the renin-angiotensin system4,5 and regional inflammatory changes, such as monocyte chemoattractant protein-1 expression,6 synthesis of growth factors in the endothelium,7 and increase in neutrophil infiltration,8 which finally leads to myocardial remodeling (hypertrophy and fibrosis) and hypertension. On the other hand, we reported that reduced plasma NO level is linearly correlated with severity of hypertensive status in patients with essential hypertension,9 suggesting that this model might mimic cardiac hypertrophy associated with clinical essential hypertension.

Recent studies including ours have shown that either extracellular signal-regulated kinase (ERK)8,10,11 or 70-kDa S6 kinase (p70S6K)8,11–13 plays a critical role in the angiotensin II–associated hypertrophic changes. We also reported that either angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II type-1 receptor antagonists (ARBs) reduce cardiac hypertrophy by differential modulation of p70S6K or ERK in this model.8

In this study, we evaluated the effects of CCBs (amlodipine and benidipine) on (1) myocardial structural changes, (2) tissue p70S6K and ERK activity, and (3) the cause-and-effect relationship between morphological changes and kinase deactivation.

Methods
All procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1985) and approved by the Osaka University Ethical Committee for Laboratory Animal Use.
S6 peptide (RRRLSSLRA, No. 12–124) was purchased from Upstate Biotechnology. Protein C/arginase A-coupled beads were obtained from Oncogene Sciences, amiodipine from Sumitomo Pharmaceuticals, and benidipine from Kyowa Hakko Kogyo. The other drugs were purchased from Sigma.

**Instrumentation**

Eighty 8-week-old male Wistar-Kyoto rats were randomly divided into 10 groups. The control group received no treatment. The L-NAME group received L-NAME (1 g/L in drinking water). The D-NAME group received D-NAME (1 g/L in drinking water), the inactive isomer of L-NAME. The L-NAME+Hyd group received both L-NAME and hydralazine (120 mg/L in drinking water).

The L-NAME+(Amlo1 or Amlo3) group, the L-NAME+(Beni3 or Beni10) group, the L-NAME+Rap group, and the L-NAME+PD group received amiodipine (1 or 3 mg/kg per day), benidipine (3 or 10 mg/kg per day), rapamycin (a potent p70S6K inhibitor; 0.5 mg/kg per day), or PD98059 (a potent ERK kinase inhibitor; 5 mg/kg per day), respectively, orally by gavage in addition to L-NAME. We decided the doses of each drug according to either our preliminary experiments (data not shown) or the previous report. Both systolic blood pressure and heart rate were measured by the tail-cuff method. Rats from all groups were housed, fed, and finally anesthetized and killed after 8 weeks, as described previously.

**Histological Examination**

Excised hearts were weighed, sectioned, and carefully scanned, as described previously. The morphometry of left ventricular myocytes was assessed, and the cross-sectional area of cardiomyocytes was measured as described previously. One hundred cells per heart were counted, and the average value was used for analysis.

**Assay for P70S6K and ERK Activities**

Specific activity of p70S6K was determined by 32P incorporation into S6 peptide in the immune complex, as described previously. Briefly, cardiac tissue (n = 5 from each group studied) was lysed in 1 mL of lysis buffer. After the protein concentration was measured, the extract was incubated with rabbit polyclonal antibody against the C terminus of p70S6K. The radioactivity was determined with the use of a liquid scintillation counter. Specific activity of ERK was determined as described previously. The experiments were performed in duplicate for each sample.

**Statistical Analysis**

Data are expressed as mean±SEM. Paired data were compared by Student t test. Comparisons of p70S6K activity, ERK activity, hemodynamic parameters such as systemic blood pressure and heart rate, left ventricular weight (LVW), and cardiomyocyte cross-sectional area were performed by ANOVA followed by modified Bonferroni multiple-comparison t test. Comparison of time course changes in systemic blood pressure was performed by 2-way repeated ANOVA followed by the multiple comparison test. A probability value of <0.05 was considered statistically significant.

**Results**

**Systemic Blood Pressure and Heart Rate**

Before treatment, systemic blood pressure was comparable among the 10 groups studied. After 8 weeks of treatment, systemic blood pressure was comparable in 6 groups but higher in the L-NAME, L-NAME+Amlo1, L-NAME+Rap, and L-NAME+PD groups (P < 0.05 each) than in the control group (Figure 1A). The increase in systemic blood pressure in this model was abolished by 3 mg/kg per day of amiodipine/benidipine or hydralazine but was further decreased in the L-NAME+Beni10 group to a value that was lower (P < 0.01) than that in the control group. Heart rate was comparable and did not change significantly among all groups throughout this study (Figure 1B).

**Left Ventricular Weight and Myocardial Hypertrophy**

After 8 weeks of treatment, all groups other than D-NAME and L-NAME+Amlo3 groups showed significantly higher LVW than the control group (Table). Amlodipine, benidipine, rapamycin, and PD98059 but not hydralazine significantly attenuated the increase in LVW induced by L-NAME (Table).

Representative histological findings of cross-sectional areas of cardiomyocytes are shown in Figure 2A. The cross-sectional areas of cardiomyocytes in the L-NAME group were significantly larger than those in the control group (P < 0.01; Figure 2B). Amlodipine, benidipine, rapamycin, and PD98059 but not hydralazine also attenuated the increase in the cross-sectional areas of cardiomyocytes induced by L-NAME (Figure 2B).

**P70S6K and ERK Activity in Cardiac Tissue**

P70S6K activity in cardiac tissue in the L-NAME group was higher than that in the control group (P < 0.01). Amlodipine, benidipine, and rapamycin but not hydralazine or PD98059 prevented the increase in p70S6K activity induced by L-NAME (Figure 3A).

<table>
<thead>
<tr>
<th>Left Ventricular Weight, mg</th>
<th>n</th>
<th>8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>780±18</td>
</tr>
<tr>
<td>LNAME</td>
<td>8</td>
<td>1048±22†</td>
</tr>
<tr>
<td>DNAME</td>
<td>8</td>
<td>807±17§</td>
</tr>
<tr>
<td>LNAME+Hyd</td>
<td>8</td>
<td>1118±29†</td>
</tr>
<tr>
<td>LNAME+Amlo1</td>
<td>8</td>
<td>829±24§</td>
</tr>
<tr>
<td>LNAME+Amlo3</td>
<td>8</td>
<td>789±27§</td>
</tr>
<tr>
<td>LNAME+Beni3</td>
<td>8</td>
<td>875±21‡</td>
</tr>
<tr>
<td>LNAME+Beni10</td>
<td>8</td>
<td>825±20§</td>
</tr>
<tr>
<td>LNAME+Rap</td>
<td>8</td>
<td>824±23§</td>
</tr>
<tr>
<td>LNAME+PD</td>
<td>8</td>
<td>845±27‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Amlo indicates amiodipine; Beni, benidipine; Hyd, hydralazine; PD, PD98059; and Rap, rapamycin. *P < 0.05 vs control; †P < 0.01 vs control; ‡P < 0.05, §P < 0.01 vs LNAME.
ERK activity in cardiac tissue in the L-NAME group was higher than that in the control group (P<0.01). Amlodipine, benidipine, and PD98059 but not hydralazine or rapamycin prevented the increase in ERK activity induced by L-NAME (Figure 3B).

Discussion

We were able to demonstrate that CCBs (amlodipine or benidipine) but not hydralazine or rapamycin prevented the increase in ERK activity induced by L-NAME (Figure 3B).

**Figure 2.** Representative histological findings of myocytes (A) and myocyte cross-sectional areas (B). *P<0.05, **P<0.01 vs control. +P<0.05, ++P<0.01 vs L-NAME group. 1, Control; 2, L-NAME; 3, D-NAME; 4, L-NAME+Hyd; 5, L-NAME+Amlodipine; 6, L-NAME+Amlodipine+Hyd; 7, L-NAME+Benidipine; 8, L-NAME+Benidipine+Hyd; 9, L-NAME+Rapamycin; 10, L-NAME+PD. Data are expressed as mean±SEM. Aml, amlodipine; Beni, benidipine; Hyd, hydralazine; PD, PD98059; Rap, rapamycin.

**Figure 3.** P70S6K activity (A) and ERK activity (B) in myocardial tissue. **P<0.01 vs control. +P<0.05, ++P<0.01 vs L-NAME group. Data are expressed as mean±SEM. Aml, amlodipine; Beni, benidipine; Hyd, hydralazine; PD, PD98059; Rap, rapamycin.

Features of Myocardial Hypertrophy Induced by Inhibition of NO Synthesis

Chronic treatment with L-NAME inhibits NO synthesis, and induces all of arterial hypertension, cardiac hypertrophy, and remodeling. However, these structural changes in the heart might be independent of arterial hypertension. Many previous reports have indicated the various mechanisms by which the inhibition of NO synthesis induces myocardial structural changes: (1) increases in plasma renin activity and local ACE activity and upregulation of angiotensin II receptors, all of which enhance the effect of angiotensin II; (2) increase in neutrophil infiltration and monocyte chemoattractant protein-1 expression; and (3) synthesis of growth factors in the endothelium. We have also demonstrated that the inhibition of NO synthesis leads to PKC activation through a cGMP-independent mechanism, which is also involved in the pathway of angiotensin II–induced cardiac hypertrophy. Taken together, it is likely that activation of the local, or in part the systemic, renin-angiotensin system and increasing inflammatory changes may contribute to cardiovascular hypertrophy in this model in both PKC-dependent and PKC-independent manners.

Mechanisms for the Modulation of P70S6K/ERK Activity by CCBs

In the present study, both rapamycin and PD98059 independently reduced myocardial remodeling. Several reports have
demonstrated that both p70S6K and ERK are independently involved in cardiac hypertrophy,\textsuperscript{13,20} supporting our current results. It is intriguing to consider how amlodipine and benidipine, long-acting dihydropyridine calcium channel blockers, modulate p70S6K/ERK activity. It has been reported that angiotensin II activates phosphoinositide-3 kinase, which then interacts with L-type calcium channel and increases peak Ca\textsuperscript{2+} current\textsuperscript{21} and increases the intracellular Ca\textsuperscript{2+} level\textsuperscript{22} in either a dihydropyridine-sensitive or dihydropyridine-insensitive manner,\textsuperscript{22} eventually leading to the activation of PKC and ERK.\textsuperscript{22} Since another report reveals that p70S6K is activated through phosphoinositide-3 kinase in cardiomyocytes\textsuperscript{23} separately from PKC or ERK,\textsuperscript{13} CCBs might regulate not only the intracellular Ca\textsuperscript{2+} level but also phosphoinositide-3 directly to modulate ERK and p70S6K, respectively. In addition, recent reports revealed that CCBs increase NO production in the heart,\textsuperscript{24} which might be attributable to the cardioprotection by CCBs in this model. Further studies on this model are expected.

**Study Limitations**

We did not test the groups treated with rapamycin only or PD98059 only. We cannot deny the possibility that these 2 drugs can further decrease LVW at the control level. However, this is not likely, since CCBs, which prevented myocardial hypertrophy in this model through modulation of both p70S6K and ERK, did not further enhance the reduction of LVW caused by rapamycin and PD98059 alone, also suggesting that complete inhibition of each kinase in this model can sufficiently inhibit hypertrophy equal to the control level.

**Perspectives**

In this study, we showed the potent subcellular signaling mechanisms of CCBs in attenuating myocardial hypertrophy apart from lowering blood pressure. We have also reported that ACEIs and ARBs regulate p70S6K or ERK differently to attenuate cardiovascular remodeling in the present model, and use in combination causes an additional effect compared with the single use of each drug.\textsuperscript{8} Therefore, CCBs might share mechanisms against cardiac remodeling with both ACEIs and ARBs, suggesting that CCBs might be more suitable agents to prevent cardiac remodeling in this model. Accordingly, even the lower doses of CCBs, especially amlodipine, cause sufficient reduction of cardiac remodeling.

However, we cannot deny other mechanisms that contribute to the effect of these drugs. Moreover, it is often difficult to block cardiac remodeling completely by various medications in the clinical setting, and intensive lowering of blood pressure per se, more marked when CCBs are used in combination with other pressure-lowering drugs such as ACEIs\textsuperscript{25} or ARBs,\textsuperscript{26} might also be beneficial in treating clinical hypertension, especially in patients with multiple risk factors.

**Acknowledgments**

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