Effects of Pitavastatin on Pressure Overload-Induced Heart Failure in Mice

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Background: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), which are widely used to lower plasma cholesterol levels, have been reported to have various pleiotropic effects such as protective effect of endothelial cells, angiogenic effect, antioxidant effect and anti-inflammatory effect. It is unclear, however, whether statins have any effects on the progression from left ventricular (LV) hypertrophy to heart failure in the established hypertrophied heart.

Methods and Results: C57BL/6 mice were treated with pitavastatin (pitava) or vehicle (control) from 2 weeks (established hypertrophy stage) after transverse aortic constriction (TAC) and the treatment was continued for 4 weeks. Pitavastatin significantly inhibited the progression from LV hypertrophy to heart failure as assessed on echocardiography. The cardiomyocyte cross-sectional area was significantly increased in the control group compared to the sham-operated mice (sham group), but it was not significantly different between the control group and the pitava group at 6 weeks after TAC. Moreover, pitavastatin induced myocardial angiogenesis (ratio of number of endothelial cells to cardiomyocytes) and decreased the myocardial fibrosis and oxidative stress. The expression of angiopoietin-1 in the heart was significantly increased by pitavastatin at 6 weeks after TAC.

Conclusions: Pitavastatin has preventive effects on the progression of heart failure even in the hypertrophied heart. (Circ J 2012; 76: 1159–1168)

Key Words: Angiogenesis; Cardiac fibrosis; Heart failure; HMG-CoA reductase inhibitor; Left ventricular hypertrophy

Left ventricular (LV) hypertrophy occurs as an adaptive response to increased workload, but prolonged LV hypertrophy results in cardiac dysfunction leading to cardiovascular events such as congestive heart failure (CHF) and sudden death. CHF is a complex syndrome that consists not only of LV dysfunction but also of metabolic and neurohumoral alterations. Despite numerous studies on LV hypertrophy and CHF, the precise mechanisms of the transition from LV hypertrophy to CHF are not fully understood. During the past 20 years there has been considerable progress in the treatment of CHF, with the introduction of angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, β-blockers and aldosterone antagonists. The number of deaths due to CHF, however, has been increasing steadily and further strategies for CHF are needed.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, so called statins, are widely used to lower plasma cholesterol levels. Clinical trials have shown that statins significantly reduce the incidence of cardiovascular events in patients with coronary artery disease. It has been reported that the beneficial effects of statins on coronary heart disease are independent of their ability to lower plasma cholesterol levels. Moreover, statins exhibit various pleiotropic effects such as improvement of endothelial function, enhancement of the stability of atherosclerotic plaques, acceleration of ischemia-induced angiogenesis, decrease in oxidative stress, suppression of inflammation and inhibition of the thrombogenic response. Furthermore, treatment with statins before the initiation of LV hypertrophy has been reported to suppress the development of LV hypertrophy. These effects of statins suggest the potential to ameliorate components of the complex pathophysiology of CHF and to contribute to a promising treatment for CHF in the future. But, it is unclear whether statins can inhibit the progression from LV hypertrophy to heart failure.
pertrophy to CHF.

The murine transverse aortic constriction (TAC) model is widely used to characterize the impact of genetic or pharmacologic interventions on LV remodeling. After TAC, LV hypertrophy progresses, and then LV function deteriorates with the symptoms of CHF. Recent studies have shown that there is a myocardial inflammatory reaction and a fibrosis response after TAC. Pitavastatin, a statin, was developed in Japan and has a potent lipid-lowering effect. Little is known about its effects on the heart. The purpose of the present study was to determine the effects of pitavastatin on the progression from LV hypertrophy to CHF in a murine TAC model.

### Methods

#### Animals

All animals were treated humanely and all experimental procedures were performed according to the guidelines established by Chiba University for experiments in animals. All protocols were approved by the institutional review board. C57BL/6 mice (8 weeks old) were purchased from SLC (Shizuoka, Japan) and used in the present study. Two weeks after TAC, cardiac function of each mouse was evaluated on echocardiography. Mice with LV hypertrophy were randomly divided into a pitavastatin (pitava) group or a vehicle (control) group. After 4 weeks of pitavastatin or vehicle, echocardiography was performed again. The levels of serum total cholesterol, triglyceride, and creatine kinase were measured.

#### Table. LV Dimension and Function on Echocardiography

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<thead>
<tr>
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<th>2 weeks after TAC</th>
<th>6 weeks after TAC</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Control</td>
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<tr>
<td><strong>FS (%)</strong></td>
<td>46.0±2.21</td>
<td>39.5±0.50</td>
</tr>
<tr>
<td><strong>IVSTd (mm)</strong></td>
<td>0.76±0.06</td>
<td>1.27±0.02</td>
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<tr>
<td><strong>PWTd (mm)</strong></td>
<td>0.75±0.03</td>
<td>1.01±0.02</td>
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<tr>
<td><strong>LVDd (mm)</strong></td>
<td>3.27±0.09</td>
<td>3.13±0.05</td>
</tr>
<tr>
<td><strong>LVDs (mm)</strong></td>
<td>1.76±0.12</td>
<td>1.91±0.05</td>
</tr>
<tr>
<td><strong>HR (/min)</strong></td>
<td>589±22</td>
<td>638±9</td>
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<tr>
<td><strong>BW (g)</strong></td>
<td>22.9±0.97</td>
<td>24.0±0.31</td>
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<tr>
<td><strong>HW/BW (mg/g)</strong></td>
<td>–</td>
<td>24.0±0.31</td>
</tr>
<tr>
<td><strong>LW/BW (mg/g)</strong></td>
<td>–</td>
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Data given as mean±SEM. 1P<0.05 vs. each group at 2 weeks. *P<0.05 vs. sham group at 6 weeks. **P<0.05 vs. control group at 6 weeks. LV, left ventricular; TAC, transverse aortic constriction; pitava, pitavastatin; FS, fractional shortening; IVSTd, intraventricular septum end-diastolic thickness; PWTd, posterior wall end-diastolic thickness; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; HR, heart rate; BW, body weight; HW, heart weight; LW, lung weight.

**Figure 1.** Representative M-mode echocardiography at 6 weeks after transverse aortic constriction. Pitava, pitavastatin.
terol (TC), creatinine (Cre) and creatine kinase (CK) at 6 weeks after TAC were determined on high-performance liquid chromatography at SRL (Tokyo, Japan). Mice were then killed for further analyses.

**TAC**

Mice were anesthetized with isoflurane, endotracheally intubated with a 24-G catheter and ventilated as reported previously. The transverse aortic arch was surgically accessed and ligated (7-0 silk strings) between the right innominate and left common carotid arteries with an overlying blunted 27-G needle, followed by prompt removal of the needle. After aortic constriction, the chest was closed and mice were allowed to recover from anesthesia. Sham-operated mice (sham) underwent the same procedure except that aortic constriction was not done.

**Drugs**

Pitavastatin (Kowa Pharmaceutical, Tokyo, Japan) was used at a dose of 3 mg·kg$^{-1}$·day$^{-1}$ in carboxymethylcellulose suspension by gavage.

**Echocardiography**

Transesophageal echocardiography was performed with a VisualSonic (Vevo 660; VisualSonics, Toronto, Canada) equipped with a 25-MHz imaging transducer. Mice were kept awake without anesthesia during echocardiography to minimize data deviation. Echocardiography was performed before TAC, and at 2 weeks and 6 weeks after TAC. To minimize variation of the data, the heart rate was always approximately 550–650 beats/min during the examination. We determined the LV end-diastolic diameter (LVDd) and the end-systolic diameter (LVDs) in the M-mode recordings. The intraventricular septum end-diastolic thickness (IVSTd) and LV posterior wall end-diastolic thickness (PWTd) were measured at the time of LVDd measurement. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = (LVDd-LVDs)/LVDd × 100.

**Histology**

Cardiac tissue was fixed by perfusion with 4% paraformaldehyde. The fixed samples were embedded in paraffin and sectioned at 4 μm thickness for hematoxylin-eosin (H&E) and Masson trichrome staining. For measurement of the cardiomyocyte cross-sectional area, 100 randomly selected cardiomyocytes in left ventricular cross-section were measured. To determine the degree of myocardial fibrosis, we selected 10 fields at random and calculated the ratio of Masson trichrome staining fibrosis area to total myocardium area with NIH IMAGE (NIH, Research Service Branch). The degree of perivascular fibrosis of arteries was calculated according to the following formula: B/A.

**Figure 2.** Effects of pitavastatin (pitava) on transverse aortic constriction (TAC)-induced cardiomyocyte hypertrophy in mice. Mice were treated with vehicle or pitavastatin from 2 weeks to 6 weeks after TAC. (A) H&E staining of the heart at 6 weeks after TAC. Bar, 150 μm. (B) Cardiomyocyte cross-sectional area, measured using 100 randomly selected cardiomyocytes in left ventricular cross-section. Data expressed as mean±SEM (n=10). **P<0.01.
Staining was scored as follows: 0, no visible staining; 1, faint staining; 2, moderate staining; and 3, strong staining.

Western Blot
At 6 weeks after TAC, whole heart lysates were subjected to western blot analysis. The extracts were prepared in lysis buffer and the total protein concentration was measured with the BCA protein assay kit (Pierce, IL, USA). Proteins (50 μg) were separated in 10–15% sodium dodecylsulfide-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, MA, USA). After blocking, the membranes were incubated with antibodies to phospho-ERK (p-ERK; Cell Signaling, MA, USA), angiopoietin-1 (Ang-1; Rockland, WI, USA), angiopoietin-2 (Ang-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology, Santa Cruz, CA, USA), fibronectin-1 (Fn1), collagen type Iα1 (Col Iα1), collagen type IIIα1 (Col IIIα1) and atrial natriuretic peptide (ANP) was performed. Total RNA was extracted from the samples using the RNeasy kit (Qiagen, Valencia, CA, USA). We used 0.5 μg of total RNA to generate cDNA using the Super Script VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out on a LightCycler system (Roche, Mannheim, Germany) using probes from Universal Probe Library and the TaqMan Master Mix. Sequence of primers and the respective Universal Probe Library probes were as follows: TGF-β: forward, TGGAGCAACATGTGGAACTC; reverse, GTCAGCAGCCGGTTACCA; Fn1: forward, CG-
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GAGAGGTGCCCCTACTA; reverse, CGATATTGGTGAATCGCAGA; Col Iα1: forward, CATGTCAGCTTTGTGGAA-CCT; reverse, GCAGCTGACTTCAGGGATGT; Col IIIα1: forward, TCCCCTGGAATCTGTGAATC; reverse, TGAGTC-GAATTGGGGAGAAT; Anp: forward, CACAGATCTGATGGATTTCAAGA; reverse, CCTCATCTTCTACCGGCATC; Gapdh: forward, TGTCCGTCGTGGATCTGAC; reverse, CCGCTTCACCACCTTCTTG. Relative expression of target genes was calculated with the comparative CT method. Each sample was run in duplicate, and the results were systematically normalized using Gapdh.

Statistical Analysis
All data are expressed as mean±SEM of 5–6 independent experiments. Mean difference among 3 groups was tested using 1-way ANOVA followed by the Bonferroni procedure for comparison of means. The difference among groups was analyzed on Student’s t-test. P<0.05 was considered statistically significant. SPSS version 18.0 (SPSS, Chicago, IL, USA) was used for all analyses.

Results
We first assessed the LV wall thickness, dimension and function on echocardiography to confirm pressure overload-induced cardiac remodeling. LV hypertrophy and dysfunction were recognized at 2 weeks after TAC (IVSTd, 0.83±0.02–1.32±0.02 mm, P<0.01; PWTd, 0.75±0.01–1.06±0.02 mm, P<0.01; FS, 46.6±0.93–39.9±0.40, P<0.01). After the assessment, mice were divided into 2 groups: control group, treated with vehicle from 2 weeks after TAC for 4 weeks; pitava group, treated with pitavastatin from 2 weeks after TAC for 4 weeks. There were no significant differences in physiological parameters such as body weight (BW), heart rate and echocardiographic parameters before treatment between the control and the pitava groups (Table). All mice in the control and the pitava groups were alive during the study. Although the thickness of the LV wall was not significantly different between the control group and the pitava group, LV systolic function significantly deteriorated in the control group but not in the pitava group at 6 weeks after TAC (FS: control, 27.6±1.52%; pitava, 40.3±2.13%; P<0.05; Figure 1; Table). The heart weight (HW)/BW ratio and lung weight (LW)/BW ratio were significantly increased in the control group compared to the sham group, but they were significantly decreased in the control group compared to the sham group, but they were significantly increased in the pitava group (HW/BW: sham, 3.90±0.07; control, 8.24±0.48; pitava, 7.33±0.38; LW/BW: sham, 5.48±0.10; control, 14.8±1.56; pitava, 9.82±1.49). There were no significant differences in serum TC level (control, 81.4±5.3 mg/dl; pitava, 81.0±2.3 mg/dl), Cre (control, 0.20±0.01 mg/dl; pitava, 0.28±0.07 mg/dl) and CK (control, 25.5±5.5 IU/L; pitava, 47.3±9.4 IU/L) at 6 weeks after TAC between the 2 groups.

Effects of Pitavastatin on Cardiomyocyte Size, Myocardium Fibrosis and Angiogenesis
To evaluate the effect of pitavastatin on the hypertrophy of cardiomyocyte, we measured the cardiomyocyte cross-sectional area. Cardiomyocyte cross-sectional area was significantly increased in the control group and the pitava group compared to the sham group, but it was not significantly dif-
different between the control group and the pitava group at 6 weeks after TAC (sham, 11,098±395 μm²; control, 14,006±542 μm²; pitava, 13,490±868 μm²; Figure 2). To determine the effect of pitavastatin on myocardium fibrosis, we next examined the fibrosis area on Masson trichrome staining. The area of total myocardial fibrosis was significantly increased in the control group compared to the sham group, but it was decreased in the pitava group (sham, 1.04±0.23%; control, 7.40±0.61%; pitava, 3.63±0.49%; Figures 3A,B). The area of perivascular fibrosis in the myocardium was significantly increased in the control group compared to the sham group, but it was decreased in the pitava group (sham, 13.7±2.55%; control, 63.3±10.16%; pitava, 33.0±3.37%; Figures 3A,C).

To determine the effect of pitavastatin on angiogenesis, we performed immunohistochemical double-staining of myocardium for PECAM and dystrophin. The ratio of PECAM-positive endothelial cells to dystrophin-positive cardiomyocytes was not changed in the control group compared to the sham group, but it was significantly increased in the pitava group (sham, 1.00±0.02; control, 0.98±0.08; pitava, 1.18±0.13; Figure 4).

**Effects of Pitavastatin on Oxidative Stress in Myocardium**

Given that statins are known to decrease oxidative stress, we...
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Effects of Pitavastatin on Expression of TGF-β, Fn1, Col Iα1, Col IIIα1 and ANP

We analyzed the expression of fibrosis-related genes such as Fn1, TGF-β, Col Iα1 and Col IIIα1 mRNA using qRT-PCR. The gene expression of Fn1, TGF-β, Col Iα1 and Col IIIα1 in myocardium was significantly increased in the control group compared to the sham group, but was significantly decreased in the pitava group (TGF-β: sham, 1.00±0.09; control, 1.54±0.13; pitava, 0.86±0.08; Fn1: sham, 1.00±0.08; control, 2.05±0.43; pitava, 1.13±0.68; Col Iα1: sham, 1.00±0.08; control, 2.78±0.30; pitava, 0.98±0.08; Col IIIα1: sham, 1.00±0.16; control, 7.81±2.89; pitava, 1.04±0.10; Figure 5A,B). On western blot, the expression of 4-HNE in myocardium was also significantly increased in the control group compared to the sham group, but it was significantly decreased in the pitava group (sham, 1.00±0.13; control, 6.97±0.77; pitava, 3.64±0.70; Figures 5C,D).

Effects of Pitavastatin on Expression of VEGF, Ang-1, Ang-2, Tie-2

To determine the mechanisms of pitavastatin in the heart, we analyzed the expression of p-ERK, Ang-1 and VEGF on western blot. The expression of p-ERK was not changed between the control group and the pitava group (sham, 1.00±0.09; control, 0.93±0.03; pitava, 1.01±0.09). The expression of Ang-1 was not changed at 6 weeks after TAC, but it was significantly increased by pitavastatin (sham, 1.00±0.30; control, 14.6±1.34; pitava, 5.54±0.48; Figure 6A). Finally, we examined the expression of ANP mRNA on qRT-PCR. The expression of ANP is known to be increased in CHF. The expression of ANP mRNA was increased at 6 weeks after TAC, but it was significantly decreased by pitavastatin (sham, 1.00±0.30; control, 14.6±1.34; pitava, 5.54±0.48; Figure 6B).
Discussion

In the present study we evaluated whether pitavastatin has any effect on the progression from LV hypertrophy to CHF in the established hypertrophied heart. The results suggest that pitavastatin has an inhibitory effect on the progression of CHF through angiogenesis.

LV hypertrophy is an independent cardiovascular risk factor in the general population, especially in hypertensive patients. A greater reduction of LV mass has been associated with a lower incidence of cardiovascular events in hypertensive patients. \(^{30,31}\) Activation of the renin-angiotensin system and an increase in angiotensin II level induce hypertrophy of cardiomyocytes. \(^{32}\) Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers are reported to inhibit the progression of LV hypertrophy and CHF. Statins have also been reported to inhibit the development of LV hypertrophy without lowering blood pressure in angiotensin II-infusion or pressure overload animal models. \(^{17,18,33}\) It remains unknown, however, whether statins have beneficial effects even on the established hypertrophied heart. In the present study, we used pitavastatin to investigate those effects of statins. Pitavastatin was developed in Japan and has potent lipid-lowering effects. \(^{34,35}\)

Treatment with pitavastatin inhibited the progression of LV hypertrophy to CHF in the pressure overload-induced hypertrophied heart. Although treatment with pitavastatin for 4 weeks did not cause the LV hypertrophy to regress, LV systolic dysfunction and the increased gene expression of ANP was inhibited by pitavastatin in the present study. LV fibrosis was decreased by pitavastatin. It has been reported that several pro-inflammatory cytokines induce LV fibrosis and that statins decrease the expression of those cytokines by anti-inflammatory effect. Therefore, there is a possibility that pitavastatin inhibits LV fibrosis through the anti-inflammatory effect. Interestingly, pitavastatin could also prevent progression from

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**Figure 7.** Effects of pitavastatin (pitava) on expression of vascular endothelial growth factor (VEGF), angiopoietin (Ang)-1, Ang-2 and Tie-2. Mice were treated with vehicle or pitavastatin from 2 weeks to 6 weeks after transverse aortic constriction (TAC). (A) Proteins were extracted from the heart of mice at 6 weeks after TAC and subjected to western blot. Equal loading was validated by the expression of GAPDH. (B) Quantitative analysis of the expression of VEGF, Ang-1, Ang-2 and Tie-2 protein in the heart. Data expressed as mean±SEM. *P<0.05, **P<0.01.
LV hypertrophy to CHF. An increase in the number of vessels is necessary for progression of LV hypertrophy. A mismatch between the number of vessels around the cardiomyocyte and the size of the cardiomyocyte leads to the transition from LV hypertrophy to CHF. The present data suggest that pitavastatin blocked the decrease in the number of vessels after TAC by increasing the expression of Ang-1. There was no significant difference in the expression of VEGF between the control group and the pitavata group at 6 weeks after TAC. In the present study, pitavastatin increased the expression of Ang-1, but did not affect the expression of Ang-2 and Tie-2. Ang-1 induces recruitment of smooth muscle cells to primitive vessels consist of endothelial cells, and thus it promotes maturation of newly formed blood vessels.36,37 Ang-1 also induces endothelial cell survival through several pathways, including PI3-kinase/Akt.38,39 Ang-1 acts as a negative regulator of VEGF-induced angiogenesis, meanwhile the Ang-1/Tie-2 signal is known to induce physiological and pathological angiogenesis.40,41 The present results suggest that pitavastatin treatment may become an approach to increase stable and functional vessels through enhancement of Ang-1 production in hypertrophied heart.

Pitavastatin is reported to improve the progression of LV hypertrophy through the inhibition of RhoA-ERK-serum response factor signaling in the Dhal rat.42 In that study the effect of pitavastatin on cardiac fibrosis and the gene expression of ANP, Fn1, TGF-β, Col Ia1 and Col IIIa1 were almost the same as in the present study, but pitavastatin inhibited the initiation of LV hypertrophy. In Dhal rats, the effect of over-intake of sodium may modify the molecular changes in the heart. In recent clinical trials (CORONA and GISSI-HF), hydrophilic statin, rosuvastatin, failed to reduce the cardiovascular events in patients with CHF.43,44 CHF patients with ischemic heart disease were included in the studies (CORONA; 100%, GISSI-HF; 40%), but the present CHF model was generated by pressure overload. The etiology of CHF might affect the cardioprotective effects of statins. Because it is reported that hydrophobic statins have more cardioprotective effects compared to hydrophilic statins,45 it is possible that hydrophobic statins and hydrophilic statins have different effects on the heart.

In conclusion, pitavastatin had cardioprotective effects in pressure overload-induced CHF. These results suggest that pitavastatin may be a novel therapeutic agent for CHF in patients with LV hypertrophy.

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Disclosures
The authors confirm that there are no conflicts of interest.

References


