Clinically Administered Doses of Pitavastatin and Rosuvastatin Effects on Myocardial Hypertrophy Using Cultured Cardiomyocytes

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Summary

Clinical studies have indicated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, can potentially inhibit chronic heart failure. In the Stat-LVDF study, a difference was noted in terms of the effect of lipophilic pitavastatin (PTV) and hydrophilic rosuvastatin (RSV) on plasma BNP, suggesting that statin lipophilicity and pharmacokinetics change the pleiotropic effect on heart failure in humans. Therefore, we assessed the beneficial effects of PTV on hypertrophy in cardiac myocytes compared with RSV at clinically used doses. Cultured cardiomyocytes were stimulated with 30 μ M phenylephrine (PE) in the presence of PTV (250 nM) or RSV (50 nM). These doses were calculated based on the maximum blood concentration of statins used in clinical situations in Japan. The results showed that PTV, but not RSV, significantly inhibits the PE-induced increase in cell size and leucine incorporation without causing cell toxicity. In addition, PTV significantly suppressed PE-induced mRNA expression of hypertrophic response genes. PE-induced ERK phosphorylation was inhibited by PTV, but not by RSV. Furthermore, PTV significantly suppressed the angiotensin-II-induced proline incorporation in primary cultured cardiac fibroblasts. In conclusion, a clinical dose of PTV was noted to directly inhibit cardiomyocyte hypertrophy and cardiac fibrosis, suggesting that lipophilic PTV can be a potential drug candidate against chronic heart failure.

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Key words: Lipophilic statin, Hydrophilic statin, Cardiomyocyte hypertrophy, Heart failure

Heart failure is a chronic condition that presents as a major health problem owing to its high morbidity and mortality rates.¹⁾ Heart failure is often complicated by multiple comorbidities, including coronary artery disease, arrhythmia, hypertension, diabetes mellitus, and renal insufficiency.²⁾ These comorbidities contribute to the pathogenesis of heart failure and may limit available treatments. For the past 25 years, most therapeutic agents have been targeted for the treatment of patients with systolic heart failure having depressed left ventricular ejection fraction (LVEF). Long-term treatment with a combination of angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, mineralocorticoid receptor antagonists, and β -adrenergic receptor antagonists has been used to improve symptoms, hemodynamics, and clinical outcomes.³⁾ Although considerable progress has been made in achieving clinical outcomes using these agents, the 5-year survival rate in patients with severe heart failure remains to be less than 50%; therefore, further development in therapy is needed.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are the most widely

used drugs for the treatment of hypercholesterolemia. The primary mechanism of action of statins is to reduce serum cholesterol by inhibiting hepatic cholesterol biosynthesis. Numerous reports have shown the low-density lipoprotein cholesterol (LDL-C)-independent pleiotropic effects of statins, which include anti-inflammatory and anti-oxidant effects, along with inhibition of endothelial dysfunction, which can be beneficial for the prevention of heart failure.⁴⁾ In fact, several clinical studies have shown that statin treatment can suppress the development of heart failure.⁴⁻⁷⁾ In a PEARL study report, lipophilic pitavastatin (PTV) treatment did not exhibit a significant effect on cardiac death and hospitalization for worsening heart failure; however, in the subgroup of patients with LVEF $\geq$ 30% administering PTV, cardiac death and hospitalization were noted to be significantly reduced.⁸⁾ A meta-analysis showed that statin treatment in patients with chronic heart failure may improve cardiac function and clinical symptoms and ameliorate the left ventricular remodeling.⁹⁾ However, 2 large, randomized, controlled trials, namely, Controlled Rosuvastatin in Multinational Trial in Heart Failure (CORONA)¹⁰⁾ and the effect of rosuvastatin in pa-

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tients with chronic heart failure (GISSI-HF),¹¹⁾ reported that rosuvastatin (RSV), which is a hydrophilic statin, is not effective in patients with heart failure.

It has been reported that statins differ in their pharmacokinetic profiles and selectivity for hepatic tissues. Lipophilic statins pass through the cell membrane mostly by passive diffusion, whereas hydrophilic statins (pravastatin and RSV) require carrier-mediated transport and are more selective for hepatic tissues than their counterparts.¹²⁾ As the pravastatin and RSV show reduced potential for uptake by peripheral cells, the pleiotropic effects of statins by non-hepatic effects of isoprenoid inhibition may be different between hydrophilic and lipophilic statins. Previously, we have compared the effects of lipophilic and hydrophilic statins on left ventricular diastolic dysfunction in a Stat-LVDF study. This study revealed that statin treatment did not significantly alter diastolic dysfunction (E/E'); however, plasma BNP was significantly increased in the RSV group, but not in the PTV group.¹³⁾ Furthermore, statins have been shown to exert preventive effects on cardiac hypertrophy and fibrosis *in vitro* and *in vivo*.¹⁴⁻¹⁸⁾ These findings prompted us to investigate the differences in the effects of lipophilic and hydrophilic statins on heart failure. The cholesterol-lowering effect is similar between these statins, but the aforementioned clinical studies suggested that the pleiotropic effect on cardiomyocytes and cardiac fibroblasts is different between lipophilic and hydrophilic statins. We hypothesize that the lipophilic PTV will show different effects on hypertrophy in cultured cardiomyocytes and on fibrosis in cardiac fibroblasts compared to hydrophilic RSV at clinically applicable doses. Therefore, this current study investigated the direct effect of lipophilic PTV and hydrophilic RSV on cardiomyocyte hypertrophy and cardiac fibrosis, which are known risk factors for heart failure, using doses applicable in clinical settings.

Methods

Material: PTV was kindly provided by Kowa (Tokyo, Japan), while RSV was kindly provided by Shionogi Co. Ltd. (Osaka, Japan).

Neonatal rat ventricular cardiomyocytes and fibroblasts: Primary neonatal rat ventricular cardiomyocytes and fibroblasts were isolated from 1- or 2-day-old Sprague-Dawley rats (SLC, Shizuoka, Japan) as previously described.¹⁹⁾ Hearts from SD rats were then removed, minced, and incubated with collagenase (Worthington, USA)/pancreatin (Sigma-Aldrich, USA) in Hank's Balanced Salt Solution (Nacalai Tesque, USA) at 37 °C. Cardiomyocytes and fibroblasts were separated using the Percoll gradient method as described in a previous study.²⁰⁾ These cells were cultured in Dulbecco's Modified Eagle medium (Nacalai Tesque Inc., Kyoto, Japan), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37 °C in a 5% CO₂ atmosphere. Animal studies were carried out according to the Guidelines for Animal Experiments, drawn up by the Committee for Ethics in Animal Experimentation of the University of Shizuoka, which meets the ethical standards required by law and the guidelines for experi-

mental animals in Japan.

Immunocytochemistry and measurement of cell surface area: Cardiomyocyte hypertrophy was measured as previously described.²¹⁾ The cardiomyocytes were grown in flask-style chambers with glass slides (Nalgen Nunc, Tokyo, Japan). The cells were then treated with or without PTV and RSV for 2 hours and then treated with 30 μM phenylephrine (PE). After culturing the cells for 48 hours, cardiomyocytes were stained with an anti-myosin heavy chain (MHC) antibody (Leica Biosystems, Germany). In total, 40 cardiomyocytes that were positively stained were randomly selected for analysis. The surface area of these cells was measured using ImageJ software (version 1.46).

In vitro growth inhibition assay: The assay was performed as previously described, with some modifications.²²⁾ The cardiomyocytes (2.0×10^3 cells) were plated in 96-well flat-bottomed plates and were cultured overnight. The cells were incubated for 48 hours in the presence of various concentrations of statins. A Cell Counting Kit-8 (CCK-8) solution (Dojindo, Tokyo, Japan) was added to each well, and the cells were further incubated for 4 hours at 37 °C before measuring the absorbance at 450 nm.

Leucine and proline incorporation: Cardiac myocyte hypertrophy and collagen synthesis were determined by [³H]-leucine and [³H]-proline incorporation, respectively, as previously described.²³⁾ [³H]-Leucine (0.5 μCi, Perkin-Elmer, Tokyo, Japan) was added to the culture medium, and the cardiomyocytes were further incubated for 6 hours. [³H]-Proline (0.5 μCi, Perkin-Elmer) was added to the culture medium, and the fibroblasts were further incubated for 48 hours. The cells were then washed three times with ice-cold phosphate-buffered saline (PBS) and incubated with 10% trichloroacetic acid for 30 minutes. Cells were then lysed in 0.5 N NaOH for 30 minutes. Lysates were then neutralized with 0.5 N HCl, and the total content from each well was subjected to scintillation counting (Beckman, USA).

RNA isolation, reverse transcription, and real-time polymerase chain reaction (PCR): Real-time PCR analysis was performed as previously described, with some modifications.²⁴⁾ Total RNA was isolated using TRIzol (Invitrogen, Tokyo, Japan), and the resulting RNA was reverse-transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Real-time PCR was performed using an ABI7500 Real-Time PCR System (Applied Biosystems, USA) using the KOD SYBR qPCR Mix (TOYOBO). The primer sequences were as follows: rat ANF Fw: AGGCCATATTGGAGCAAATC, Rv: CATCTTCTCCTCCAGGTGGT; rat BNP Fw, GATTCTGCTCCTGCTTTTCC; Rv, CATCGTGGATTGTTCTGGAG; rat Sk-Actin Fw: AGCTGAACGTGAGATTGTGC, Rv: TAGCTCTTTTCCAGGGAGGA, rat 18S rRNA Fw: CTTA GAGGGACAAGTGCGG; Rv: GGACATCTAAGGGCAT CACA. Relative real-time PCR was performed using the comparative quantitation method.

Western blotting: Western blotting was performed as previously described²⁵⁾ with some modifications. The cells were washed with ice-cold PBS and lysed with M-PER (Pierce, Tokyo, Japan) containing protease and phosphatase inhibitors. Protein concentrations were determined

A

B

Figure 1. Effect of PTV and RSV on cardiomyocyte hypertrophy. **A:** Primary cultured cardiomyocytes were treated with pitavastatin (PTV, 10–250 nM) or rosuvastatin (RSV, 2–50 nM), and then these were stimulated with phenylephrine (PE, 30 μM). The cardiomyocytes were immunostained with anti-β-MHC antibody (**a**: non-treatment, **b**: PE, **c**: PE + PTV 250 nM, **d**: PE + RSV 50 nM). Scale bars represent 20 μm. **B:** Cell surface area was measured using ImageJ software. Data are shown as the mean ± SEM ($n = 40$ cells). Significant differences are shown as follows: *** $P < 0.001$ versus group 1, ### $P < 0.001$ versus group 4, * $P < 0.05$ between groups 7 and 10, and NS indicates not significant versus group 4.

Figure 2. Effect of leucine incorporation on cardiomyocytes. Primary cultured cardiomyocytes were treated with pitavastatin (PTV, 250 nM) or rosuvastatin (RSV, 50 nM) and then stimulated with phenylephrine (PE, 30 μ M). The cardiomyocytes were then added to [³H]-leucine. The cells were solubilized, and the radioactivity was measured using liquid scintillation counter. Data are shown as the mean $\pm$ SEM ($n = 4$). Significant differences are shown as follows: *** $P < 0.001$ versus group 1, # $P < 0.01$ versus group 4, and NS indicates not significant versus group 4.

Figure 3. Effect of PTV and RSV on cell viability. The cardiomyocytes were treated with PTV and RSV for 48 hours. Cell viability was measured using a CCK-8 kit. Data are shown as the mean $\pm$ SEM ($n = 3$). NS indicates not significant versus group 1.

using Bradford method (Pierce). The protein samples were then mixed with SDS-PAGE sample buffer (2% SDS, 10% glycerol, 6% 2-mercaptoethanol, 50 mM Tris-HCl; pH 6.8), and an equal amount of protein in each sample was subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Millipore, Tokyo, Japan) and blocked with 5% skim milk in TBST (0.9% NaCl, 0.1% Tween[®] 20, 20 mM Tris-HCl; pH7.4). Antibodies against pERK (Ser/Thr 202/204), ERK (Cell Signaling Technology, Tokyo, Japan), and GAPDH (Protein-tech, Tokyo, Japan) were used as the primary antibodies,

whereas the horseradish peroxidase (HRP)-conjugated antibodies (Sigma, Tokyo, Japan) were used as secondary antibodies. The PVDF membrane was developed using an ECL prime reagent (GE Healthcare, Tokyo, Japan). The band density was measured using ImageJ software (ver. 1.53e).

Statistical analysis: Data are reported as mean $\pm$ SEM. Significant differences were analyzed using analysis of variance with Dunnett's or Tukey's *post-hoc* tests using GraphPad Prism software (Version 6.0). Differences were considered statistically significant at $P < 0.05$.

Results

Effect of PTV and RSV on cardiomyocyte hypertrophy: Statins have been reported to exert an effect on cardiac hypertrophy *in vitro* and *in vivo*.^{17,18)} However, in reported clinical studies, only PTV has been determined to be effective on LVEF in patients. We hypothesized that the differences were due to the doses of the drugs used in the experiments. Pharmacokinetic studies in humans have revealed that the maximum concentrations of PTV and RSV are approximately 120 ng/mL and 20 ng/mL, respectively.^{26,27)} Therefore, we investigated the effects of statins on cardiomyocyte hypertrophy at these concentrations. As per our findings, PTV significantly inhibited PE-induced hypertrophy in cardiomyocytes (Figure 1A and B), but RSV did not occur at the investigated doses. In addition, compared to the maximum concentration of RSV, PTV significantly suppressed hypertrophy.

Effect of leucine incorporation on cardiomyocytes: During cardiac hypertrophy, amino acid incorporation into cardiomyocytes has been shown to increase.²³⁾ Therefore, we examined the effect of incorporating leucine into cardiomyocytes treated with PTV or RSV. As shown in Figure 2, PE was able to enhance the uptake of [³H]-leucine into cells, and the uptake was significantly suppressed by the treatment with PTV but not with RSV. None of the statins had a significant effect on cell viability, suggesting that the inhibitory effect was not dependent on cell toxicity (Figure 3).

Effect of PTV and RSV on hypertrophic gene expression: Fetal gene expression is induced in hypertrophic cardiomyocytes.²⁸⁾ Consequently, we examined PE-induced gene expression of ANF, BNP, and Sk-Actin after treatment with statins. Gene expression was significantly increased following PE treatment. PTV, but not RSV, significantly inhibited gene expression in cardiomyocytes at the doses used in clinical practice (Figure 4).

Effect of PTV and RSV on ERK: The GTP-binding proteins (Ras, Rho, and Rac) have been determined to be important signal regulators in the mechanism by which statins exert pleiotropic effects on cardiomyocytes.^{29,30)} ERK activation, a downstream signaling mediator of Ras, is an important signaling pathway for cardiomyocyte hypertrophy, and statins have been reported to inhibit ERK activation in cardiomyocytes.^{17,31)} We examined PE-induced ERK activation after statin treatment. ERK activation was increased by PE treatment; however, PTV, but not RSV, remarkably inhibited PE-induced activation in cardiomyocytes at the investigated doses (Figure 5).

Figure 4. Effect of PTV and RSV on hypertrophic gene expression. Primary cultured cardiomyocytes were treated with pitavastatin (PTV, 250 nM) or rosuvastatin (RSV, 50 nM), and then these were stimulated with phenylephrine (PE, 30 μ M). The total RNA was isolated from the cardiomyocytes and was reverse-transcribed. Real-time PCR analysis was performed and relative gene expression was calculated using the $\Delta\Delta$ Ct method. Data are shown as the mean $\pm$ SEM ($n = 3$ or 4). Significant differences are shown as follows: * $P < 0.05$ versus group 1, # $P < 0.05$ versus group 2. NS indicates not significant versus group 2.

Effect of PTV and RSV on cardiac fibrosis: Cardiac fibrosis is an essential process for cardiac remodeling,³¹⁾ and statins have been reported to inhibit cardiac fibrosis.^{17,32,33)} Proline uptake is an indicator of collagen synthesis in an *in vitro* experimental model. Ang II-induced proline incorporation was examined in cardiac fibroblasts after statin treatment. Ang II-induced proline incorporation was remarkably inhibited by PTV, but not by RSV, in cultured cardiac fibroblasts at clinically applicable doses (Figure 6).

Discussion

In this study, we have demonstrated how PTV suppresses cardiomyocyte hypertrophy more effectively than RSV in clinically used doses, indicating that this is one of the possible causes of the differences in the results observed in clinical trials. Statins lower the serum cholesterol levels in patients with hyperlipidemia, but it is increasingly being recognized that these drugs have a pleiotropic effect on inflammation, oxidative stress, and vascu-

lar performance.⁴⁾ The pleiotropic effects of statins are considered beneficial for the treatment of cardiac hypertrophy and heart failure, and previous experimental studies have shown that statin treatment suppresses cardiac hypertrophy and inhibits heart failure *in vitro* and *in vivo*.^{17,18,34)} Statins have been proposed to inhibit the downstream signaling molecules of the mevalonate pathway, particularly the Rho and Rac families.³⁵⁾ The inhibition of these small GTPases has been associated with the promotion of cardiac hypertrophy.^{36,37)} It seems that both PTV and RSV inhibit signaling, suggesting that the differences in the anti-hypertrophic effect are not dependent on the target molecules or pathways.

There are two types of statins, that is, lipophilic (such as simvastatin, atorvastatin, and PTV) and hydrophilic (such as pravastatin and RSV). The characteristics of these statins are different, including cell permeability and pharmacokinetics.^{38,39)} Some clinical studies and meta-analyses have shown that lipophilic statins improve cardiac function.^{9,40)} In contrast, RSV, which is a hydrophilic and strong statin, failed to improve cardiac function in

Figure 5. Effect of PTV and RSV on ERK. Primary cultured cardiomyocytes were treated with pitavastatin (PTV, 250 nM) or rosuvastatin (RSV, 50 nM), and these were then stimulated with phenylephrine (PE, 30 μ M) for 10 minutes (A). The cells were solubilized, and the lysates were used in western blot (B). The band density was measured using ImageJ software (ver. 1.53e). Data are shown as the mean $\pm$ SEM ($n = 5$). NS indicates not significant versus group 2. Significant differences are shown as follows: * $P < 0.05$ versus group 1, # $P < 0.05$ versus group 2.

large clinical studies such as CORONA and GISSI-HF.^{10,11)} Lipophilic statins likely produce better survival and other outcome benefits compared to hydrophilic statins. Lipophilic and hydrophilic statins similarly decrease serum cholesterol, but it has been reported that pleiotropic effects of these types of statins vary,^{39,41)} suggesting that the usefulness of lipophilic statins in heart failure results from pleiotropic effects, especially on cardiomyocytes. RSV was reported to show direct anti-hypertrophic effects on

Figure 6. Effect of PTV and RSV on cardiac fibrosis. Primary cultured cardiac fibroblasts were treated with pitavastatin (PTV, 250 nM) or rosuvastatin (RSV, 50 nM) and then stimulated with angiotensin II (Ang II, 100 nM). The culture was then added to [3 H] proline. The cells were solubilized, and the radioactivity measured using liquid scintillation counter. Data are shown as the mean $\pm$ SEM ($n = 4$). Significant differences are shown as follows: * $P < 0.05$ versus group 1, # $P < 0.05$ versus group 2. NS indicates not significant versus group 2.

cardiomyocytes in previous studies, which has been agreement with our study results.^{17,34)} This effect occurs at high concentrations (> 100 nM), which seem to be too high for clinical use, as the clinical study reported that the C_{max} following administration of RSV was approximately 20 mg/mL (40 nM). Indeed, in our study, treatment with 50 nM RSV did not show a significant effect on cardiomyocyte hypertrophy. In contrast, the C_{max} after administration of PTV has been reported to be approximately 150 mg/mL (250 nM), and treatment with PTV at 250 nM was noted to significantly suppress hypertrophic responses in cardiomyocytes. A PEARL study revealed that PTV improves the composite of cardiac death and hospitalization due to worsening heart failure in patients with LVEF $\geq 30\%$; however, there was no significant difference in patients with LVEF $< 30\%$.⁸⁾ Therefore, lipophilic statins, such as PTV, may be considered a useful pharmacological therapy for heart failure, especially in patients with low-grade heart failure.

In conclusion, we have demonstrated that lipophilic PTV suppresses cell hypertrophy more efficiently than hydrophilic RSV in cultured cardiomyocytes using clinically relevant doses. Our data support the hypothesis that the differences in the pharmacokinetics and cell selectivity of statins are important factors for the pleiotropic effects of heart failure. This suggests that PTV can directly act on cardiomyocytes and cardiac fibroblasts, an activity assumed to play an important role in the improvement of heart failure.

Disclosure

Conflicts of interest: The authors have the following con-

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