Potential effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition on G protein-mediated cardiac hypertrophy

Eui-Young Choi

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Namsik Chung

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This certifies that the Doctoral Dissertation of is approved.

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Thesis Supervisor : Professor Namsik Chung

_____

Professor Yangsoo Jang: Thesis Committee Member

_____

Professor Ki-Chul Hwang:Thesis Committee Member

_____

Professor Jong Eun Lee: Thesis Committee Member

_____

Professor Seok-Min Kang: Thesis Committee Member

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Potential effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition on G protein-mediated cardiac hypertrophy

Eui-Young Choi

Department of Medicine

The Graduate School, Yonsei University

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Statins have recently been shown to produce anti-cardiac hypertrophic effects via the regulation of small GTPases. However, the effects of statins on G-protein mediated cardiac hypertrophy, which is the main pathway of cardiac hypertrophy, have not yet been studied. We sought to evaluate whether statin treatment directly suppresses cardiac hypertrophy through a large G-protein-coupled pathway regardless of the regulation of small GTPases. Using neonatal rat cardiomyocytes we evaluated norepinephrine (NE)-induced cardiac hypertrophy for its suppressibility by rosuvastatin

and the pathways involved by analyzing total protein/DNA content, cell surface area, immunoblotting and RT-PCR for signal transduction molecule. Treatment with NE induced cardiac hypertrophy accompanied by G_h expression and membrane translocation. Rosuvastatin inhibited G_h protein activity in cardiomyocytes by inhibiting basal and NE-stimulated mRNA transcription, protein expression and membrane translocation; however, NE-stimulated G_q protein expression was not inhibited. In a concentration-dependent manner, rosuvastatin inhibited total protein synthesis and downregulated basal and NE-induced expression of myosin light chain2 and the c-fos proto-oncogene in cardiomyocytes. In addition, the NE-stimulated PKC-MEK1,2-ERKs signaling cascade was inhibited by pretreatment with rosuvastatin. Rosuvastatin treatment also helped maintain expression levels of SERCA2a and intracellular calcium concentration. G_h protein is a novel target of statins in myocardial hypertrophy. Statin treatment may directly suppress cardiac hypertrophy through a large G_h-protein-coupled pathway regardless of the regulation of small GTPases.

Key Words : cardiac hypertrophy, G protein, statin.

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I. INTRODUCTION

Cardiac myocyte hypertrophy involves changes in cell structure and alterations in protein expression that are regulated at both the level of transcription and translation[1, 2]. There are three types of cardiac hypertrophy: normal growth, growth induced by physical conditioning (i.e.,

physiologic hypertrophy), and growth induced by pathologic stimuli[3]. Recent evidence suggests that normal and exercise-induced cardiac growth is regulated in large part by the growth hormone/insulin-like growth factor axis via signaling through the PI3K/Akt pathway [3,4]. In contrast, pathological or reactive cardiac growth is triggered by autocrine and paracrine neurohormonal factors such as epinephrine, norepinephrine (NE), angiotensin II, and aldosterone that are released during biomechanical stress and signal through the Gq/phospholipase C (PLC) pathway, leading to an increase in cytosolic calcium and the activation of protein kinase-C (PKC)[5-7].

Hypertrophic G protein-coupled receptor (GPCR) agonists such as endothelin-1(ET-1) and phenylephrine stimulate a number of protein kinase cascades in the heart [8-10]. The mitogen-activated protein kinase (MAPK) superfamily includes three major pathways: the extracellular regulated kinase (ERK1/2) pathway and two stress activated protein kinase pathways, c-Jun-NH_2-terminal kinase (JNK) and p38 MAPK [1, 11]. Heterotrimeric G protein-coupled receptors serve to convey extracellular biochemical signals to intracellular effectors. There are currently four classes of G_α proteins identified, α_s, α_i, α_{12}, and α_q. *In vitro* studies have

suggested a pivotal role for G_q-coupled receptor signaling in promoting cardiomyocyte hypertrophy. In cardiac myocytes GPCR agonists such as angiotensin II, ET-1, phenylephrine, and isoproterenol activate various levels of MAPK pathways [13]. It has been shown that under normal conditions ß-adrenergic receptors (AR) are the primary mediators of the effect of catecholamines, whereas the α1- adrenergic receptor plays a role during pathological development, such as ischemia, possibly acting as a reserve receptor system to maintain cardiac function. It was recently shown that NE induces hypertrophy in neonatal rat cardiomyocytes through α_1-AR stimulation and that G_h is partly involved in NE-induced ERKs activation [14, 15]. G_h-coupled receptors are linked to the MAPK cascade just as G_i-, G_s-, and G_q-coupled receptors are linked to the Ras–MAPK cascade. Activation of G_h-mediated ERKs is completely inhibited by calreticulin[15]. Even though α_1-ARs predominantly interact with G_q, which leads to the activation of PLC, hydrolysis of phosphoinositides, activation of PKC and mobilization of intracellular Ca^{2+}, the selectivity of the various α_1-AR subtypes for different G proteins is not clearly understood. It has been shown that NE strongly induces cardiac hypertrophy. Most experiments identifying the effects of α_1-adrenergic

stimulation on cardiac hypertrophy have been conducted in cultured cardiomyocytes from both neonates and adults. In cultured neonatal cardiomyocytes, the direct parameters related to cardiac hypertrophy are protein content and increased cell size.

A number of *in vitro* and *in vivo* studies have shown that low-molecular-weight GTPases (Rac1, Ras and Rho) are involved in the regulation of cardiac hypertrophy[2, 16, 17]. Ras and Rac1 GTPases are prohypertrophic, whereas RhoA may play only a limited role in the hypertrophic program of cardiomyocytes[18]. The 3-hydroxy-3-methylglutaryl coenzyme A (HMGCo A) reductase inhibitors, or statins, have been shown to inhibit cardiac hypertrophy and improve symptoms of heart failure by cholesterol-independent mechanisms[19-21]. Statins block the isoprenylation and function of members of the Rho guanosine triphosphatase family, such as Rac1 and RhoA[22]. Because Rac1 is a requisite component of reduced nicotinamide adenine dinucleotide phosphate oxidase, which is a major source of reactive oxygen species in cardiovascular cells, the ability of statins to inhibit Rac1-mediated oxidative stress contributes greatly to their inhibitory effects on cardiac hypertrophy. Furthermore, the inhibition of RhoA by statins leads to the

activation of protein kinase B/Akt and the up-regulation of endothelial nitric oxide synthase in the endothelium and heart[19, 23], resulting in increased angiogenesis and myocardial perfusion, decreased myocardial apoptosis, and improvement in endothelial and cardiac function. However, the effects of statins on G-protein mediated cardiac hypertrophy, which is the main pathway of cardiac hypertrophy, have not yet been studied. Therefore, in this study, NE was used to induce neurohormonal stimulation of stress-mediated or reactive cardiac hypertrophy (i.e. pathological hypertrophy). We sought to evaluate whether neurohormonal-stimulated stress could induce cardiac hypertrophy. If so, statins are likely to suppress cardiac hypertrophy. Furthermore, we also sought to evaluate whether statin treatment directly suppresses cardiac hypertrophy through a large G-protein-coupled pathway (such as G_h, G_q mediated MAPK) regardless of the regulation of small GTPases.

II. MATERIALS AND METHODS

1. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by enzymatic methods. Briefly, hearts of 1 to 2-day-old Sprague–Dawley rat pups were dissected, and the ventricles were treated with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors the hearts were minced until the pieces were approximately 1 mm^3 and treated with 10 ml of collagenase I (0.8 mg/ml, 262 units/mg, Gibco BRL) for 15 min at 37 °C. The supernatant was then removed, and the tissue was treated with fresh collagenase I solution for an additional 15 min. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes were centrifuged at 1200 rpm for 4 min at room temperature, and the cell pellet was resuspended in 5 ml of cell culture medium. The above procedures were repeated 7-9 times until little tissue was left. Cell suspensions were collected and incubated in 100-mm tissue culture dishes for 1 h to reduce fibroblast contamination. The non-adherent cells were collected and seeded

to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4–6 h, the cells were rinsed twice with cell culture medium, and 0.1 mM BrdU was added. Cells were then cultured in a CO_2 incubator at 37 °C. For stimulation with NE (10^{-5} M), the confluent cells were rendered quiescent by culturing them for 12 h in 1% (v/v) FBS instead of 10% FBS.

2. Quantification of total protein and DNA from neonatal rat cardiomyocytes

To further confirm whether there were any discrepancies between signal molecule activation and hypertrophic responses, total protein/DNA ratios were measured in cardiomyocytes after stimulation with NE for 12 h in the presence of rosuvastatin (1 μmol/L) or without rosuvastatin for an additional 24 h. Total protein/DNA ratios were measured after solubilizing the cells in 1N NaOH at 60 °C for 30 min. Total protein content was determined with BCA protein reagent (Pierce Biotechnology, IL, USA) with a bovine albumin standard according to the manufacturer's direction. For the quantitative measurement of DNA, cells were lysed by adding SDS and proteinase K, and the extraction of DNA was performed with phenol. The absorbance of the purified DNA was measured at 260 nm.

3. Confocal microscopy and fluorescence measurements

The measurement of the cytosolic free Ca^{2+} concentration was estimated by confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on a 4-well slide chamber coated with 1.5% gelatin for 1 day in α-MEM containing 10% fetal bovine serum (Gibco BRL, Paisley, UK) and 0.1 μM BrdU (Sigma Chemical, MO, USA). After incubation the cells were washed with modified Tyrode's solution with 0.265 g/L $CaCl_2$, 0.214 g/L $MgCl_2$, 0.2g/L KCl, 8.0g/L NaCl, 1g/L glucose, 0.05g/L NaH_2PO_4, and 1.0 g/L $NaHCO_3$. Cells were then loaded with 5 mM of the acetoxymethyl ester of fluo-4 (Fluo-4 AM, Molecular Probes, CA, USA) for 20 min in the dark and at 37°C. Fluorescence images were collected using a confocal microscope (Leica, Solms, Germany) excited by the 488-nm line of argon, and the emitted light was collected through a 510-560 nm band-pass filter. Relative data of intracellular Ca^{2+} were determined by measuring fluorescent intensity.

4. Immunocytochemistry

Cells were grown on 4-well plastic dishes (SonicSeal Slide, Nalge Nunc,

Rochester, NY, USA). Following incubation the cells were washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in PBS containing 0.1% triton X-100. The cells were then blocked in PBS containing 10% goat serum and incubated for 24 hr at 4℃ with rabbit polyclonal cardiac troponin T antibody. The cells were rewashed three times for 10 min with PBS and incubated with FITC-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville, NY, USA). All images were rendered using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp.). The cell surface area was measured for the evaluation of cardiac hypertrophy. One hundred cells from randomly selected fields in three wells were examined for each condition.

5. Immunoblot analysis

Immunoblot analysis for ERKs and MEK was conducted because the activation of ERKs and MEK plays an important role in gene regulation and is a sensitive and quantitative marker for the hypertrophic responses of cardiac myocytes in the mechanisms of cardiac hypertrophy. Proteins were separated by SDS-PAGE using 10–12% polyacrylamide gels and then electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were rinsed twice with water and blocked by incubation with 5% nonfat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). After 1 h of incubation at room temperature the membranes were probed overnight at 4 °C with polyclonal antibodies against phosphor-ERKs and MEKs followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were detected using an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech.). For expression analysis of Gh, the membranes were probed with anti-Gh antibodies. Additionally, membranes were probed with antiphopho-PKC to confirm the pathway.

6. RT-PCR analysis

We analyzed not only the mRNA expression levels of the protooncogenes *c-fos, c-myc, and c-jun*, which are markers of the hypertrophic response, but also those of the G proteins G_s, G_i, G_h and G_q and MLC-2v, a contractile element, and SERCA2a, Ca^{2+} regulating protein by the reverse transcription polymerase chain reaction (RT-PCR) technique in order to reveal the effects of rosuvastatin on hypertrophic mechanisms. For the RNA preparation, quiescent cardiomyocytes were treated with norepinephrine (0–100 nM) for 72 h at 37 °C in DMEM containing 0.5% serum. Total RNA was prepared with the Ultraspec-II™ RNA system (Biotecx Laboratories Inc., USA) and single-stranded cDNA was then synthesized from the isolated total RNA by AMV reverse transcriptase. A reverse transcription reaction mixture containing 1 µg of total RNA, 1X reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 units of RNAse inhibitor, 0.5 mg of oligo$(dT)_{15}$, and 15 units of AMV reverse transcriptase were incubated at 42 °C for 15 min, heated to 99 °C for 5 min, then incubated at 0–5 °C for 5 min. PCR was performed for 35 cycles with 3′- and 5′-primers based on the sequences of the c-fos gene primers; 5′-ACCATGATGTTCTCGGGTTTCAA-3′ and 5′-

CTCTGTAATGCACCAGCTCAGTCA-3′; c-myc gene primers; 5′-GAAGTGACCGACTGTTCTATGACT-3′ and 5′-CGCAACCAGTCAAGTTCTCAAGTT-3′; c-jun gene primers; 5′-AACGACCTTCTACGACGATG-3′ and 5′-GCAGCGTATTCTGGCTATGC-3′; G_s gene primers; 5′-AACAGTAAGACCGAGGACCA-3′ and 5′-AGATGATGGCAGTCACATCA-3′; G_i gene primers; 5′-CTCTAAGATGATCGACAAGA-3′ and 5′-CATGCGATTCATCTCCTCAT-3′; G_h gene primers; 5′-TTTTAAGCTTCCCGACCATGGCCGAGG-3′ and 5′-TTTTGGTACCTTAGGCGGGGCCAA-3′; and Gq gene primers; 5′-TCATTAAGCAGATGAGGATC-3′ and 5′-CTCCACAAGAACTTGATCGT-3′. For the MLC-2 gene, the primers were 5’-CGG AAG CTC CAA CGT GTT CT and 5’-TCC TTC TCT TCT CCG TGG GT and SERCA2a gene, the primers were 5′-CCATCTGCCTGTCCAT-3' and 5′-GCGGTTACTCCAGTATTG-3′.

7. Statistical analysis

All data are presented as a mean ± S.D. Data were analyzed by one-way

ANOVA followed by Tukey's Multiple Comparison Test. *P* values of <0.05 were considered significant.

III. RESULTS

1. Selectivity of adrenoceptors in NE-stimulated cardiomyocytes

To confirm the selectivity of adrenoceptors in NE-stimulated cardiomyocytes, cardiomyocyte protein synthesis was measured as an index for the hypertrophic phenotype caused by NE. Cardiomyocytes were treated with an α_1 selective antagonist, prazosin (100nM), and a β antagonist, propranolol (2μM), for 30 min, followed by NE (10μM) treatment for 24 h. NE significantly increased the protein/DNA ratio by 30% over that of the control. The α_1-antagonist, prazosin, decreased the protein/DNA ratio that was increased by NE treatment, while the β-antagonist, propranolol, did not affect the NE-induced protein/DNA ratio (Figure 1A). The phosphorylation of ERKs also was significantly increased by 3.4 fold, in cardiomyocytes stimulated by NE treatment. The phosphorylation of ERKs was especially inhibited by prazosin, as seen when compared with the control (Figure 1B). These data indicate that α_1-AR was the main mediator of the hypertrophic response in NE-stimulated neonatal cardiomyocytes.

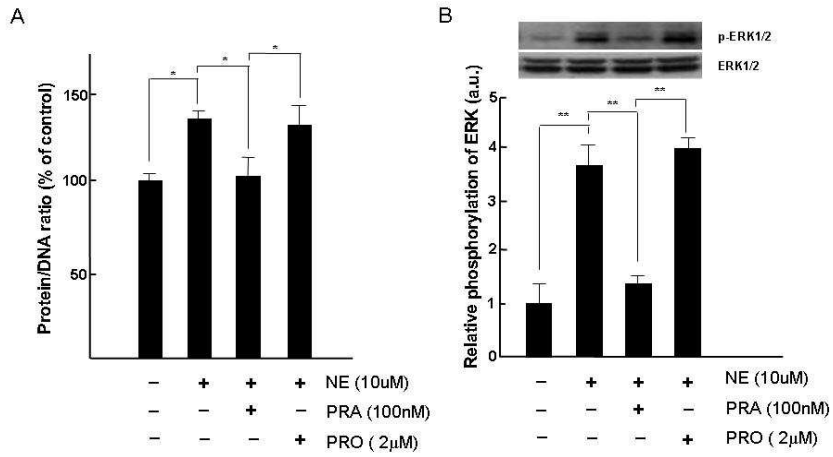

Figure 1. Selectivity of adrenoceptors in NE-stimulated cardiomyocyte s. A. Norepinephrine (NE) significantly increased the protein/DNA ratio by 30% over that of the control. While the α_1-antagonist prazosin (PRA) decreased the protein/DNA ratio that was increased by NE treatment, the β-antagonist propranolol (PRO) did not affect the NE-induced protein/DNA ratio. B. NE treatment also significantly increased the phosphorylation of ERKs by 3.4 fold in cardiomyocytes and the phosphorylation of ERKs was specifically inhibited by prazosin when compared with the control. *$p<0.05$, **$p<0.01$.

2. Rosuvastatin decreases protein content, surface area, and myosin light chain 2 mRNA expression.

To determine the effects of NE and rosuvastatin on cellular hypertrophy, cardiomyocytes were treated with NE (10 μM, 24h) and rosuvastatin (0.1–

1 µM, 36 h). NE increased the cellular protein content by 30% (Fig. 2). This increase was completely inhibited by 0.1 and 1µM rosuvastatin. Myosin light chain 2 (MLC2v) has been described as a marker of the hypertrophic phenotype. Rat neonatal cardiomyocytes treated with NE (10 µM, 24h) increased MLC2v mRNA expression by 37% (Fig. 3). Treating stimulated cardiomyocytes with rosuvastatin for 30 h resulted in decreased MLC2v expression and the downregulation of MLC2v mRNA by 10±9% and 30±19%, respectively. Treatment with rosuvastatin (0.1-1 µM) markedly inhibited the effects of NE that were seen in hypertrophy in cultured cardiomyocytes (Fig 3). The cell surface area of cardiomyocytes increased after 10µM NE treatment by 101 ± 40% and decreased to control level (110 ± 45% of control) after 1µM of rosuvastatin treatment (Fig 4.)

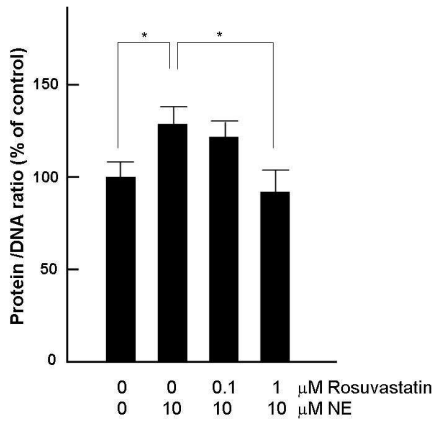

Figure 2. Inhibitory effect of Rosuvastatin on cellular protein contents in NE-stimulated cardiomyocytes. Norepinephrine increased cellular protein content by 30%. This increase was completely inhibited by 1μM rosuvastatin. *$p<0.05$.

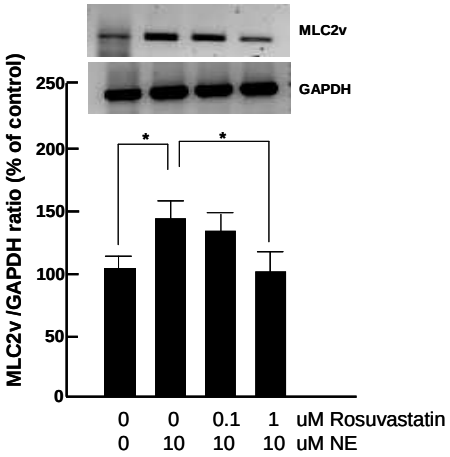

Figure 3. Inhibitory effect of Rosuvastatin in norepinephrine-induced MLC2v. Treatment with norepinephrine (10 µM, 24h) increased myosin light chain 2v (MLC2v) mRNA expression by 37%. Rosuvastatin at 0.1µM and 1µM downregulated MLC2v mRNA by 10±9% and 30±19%, respectively. *p<0.05.

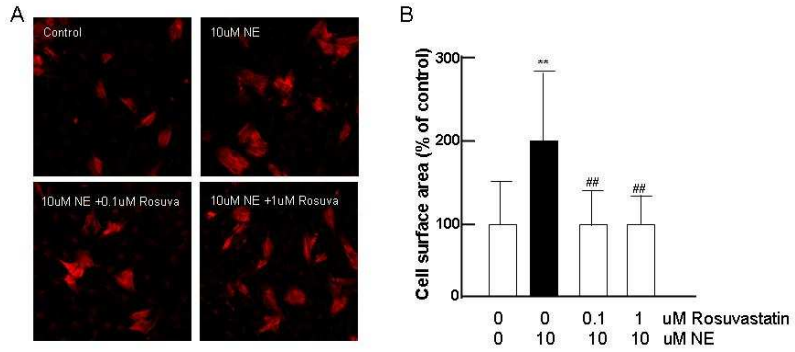

Figure 4. Inhibitory effect of Rosuvastatin on cell surface area in NE-stimulated cardiomyocytes. Immunofluorescence microscopy showed that the cell surface area of cardiomyocytes increased after 10uM of norepinephrine treatment to 201 ± 80% and decreased to 110 ± 45% after 1uM of rosuvastatin treatment. These data represent the mean ± S.D., number of wells=3-5, **p<0.01 vs. control, ##p<0.01 vs. norepinephrine.

3. Rosuvastatin inhibits G protein expression and membrane translocation.

The effect of rosuvastatin on the mRNA expression of G proteins in neonatal rat cardiomyocytes was determined by RT-PCR and western blotting. Treatment with NE (10 μM, 24 h) increased G_h and G_q expression

by 62% (Fig. 5). Pretreatment with rosuvastatin (1 µM) for 12 h significantly decreased basal G_h and G_q protein expression and inhibited the effect of NE by 42±4.3% and 40±6.8%, respectively. RT-PCR analysis after stimulation with NE (10 µM, 24h) showed upregulation of both G_h protein mRNA and G_q protein mRNA expressions by 53±16% and 54 ±15%. Treatment with rosuvastatin (1 µM, 36h) almost completely inhibited NE stimulated G_h mRNA expression to levels close to those seen in the control. However, expression of G_i and G_s mRNA was not significantly increased by NE stimulation. The function of G_h as a receptor-coupled G protein depends on both its intracellular and extracellular environments. Therefore, G_h expression was studied in both membrane and cytosolic preparations. Treatment with NE (10 µM, 24 h) increased G_h expression located in membrane by 50±20% and G_h expression in cytosol by 48±15% (Fig. 6). Pretreatment with rosuvastatin (1 µM) for 36 h decreased basal G_h expression and NE-stimulated membrane G_h expression to 120% of control levels. G_h in the cytosol was downregulated by 60±15%.

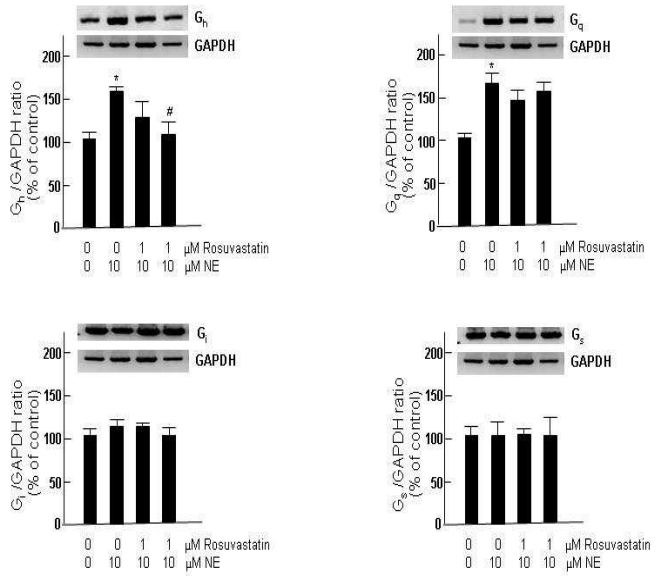

Figure 5. Effect of Rosuvastatin on G protein expression levels. RT-PCR analysis after stimulation with NE (10 µM, 24h) showed upregulation of both G_h protein mRNA and G_q protein mRNA expressions by 53±16% and 54 ±15%. Treatment with rosuvastatin (1 µM, 36h) almost completely inhibited basal G_h expression as well as NE stimulated G_h mRNA expression to levels close to those seen in the control. However, expression of G_i and G_s mRNA was not significantly increased. *$p < 0.05$ vs. control, #$p < 0.05$ vs. norepinephrine.

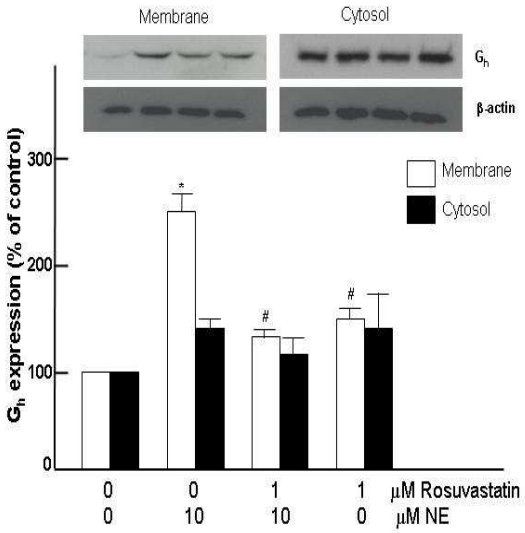

Figure 6. Inhibitory effects of Rosuvastatin on membrane translocation of G_h protein. Treatment with norepinephrine (NE, 10 μM, 24 h) increased G_h membrane expression by 150±20% and G_h cytosolic expression by 48±15%. Treatment with rosuvastatin (1 μM) for 36 h decreased NE-stimulated G_h membrane expression to 120% of control levels. G_h in the cytosol did not significantly changed. *$p<0.05$ vs. control, #$p<0.05$ vs. norepinephrine.

4. Rosuvastatin downregulates upstream regulators of ERKs in cardiomyocytes.

Increased G_h protein levels that were induced by NE affected the

hypertrophic marker ERKs in neonatal cardiomyocytes. The phosphorylation of ERKs was up-regulated close to 200 % by NE treatment (10 μM, 10min) but decreased by pretreatment with rosuvastatin (1 μM, 12 h) (Fig. 7). The phosphorylation levels of MEK, an upstream regulator of ERKs, and PKC were also significantly decreased by rosuvastatin treatment. These results showed that the intracellular signaling pathway induced by NE was primarily processed by the PKC/MEK1,2/ERKs cascade through an α_1-AR in cardiomyocytes and the PKC/MEK/ERKs cascade was directly inhibited by rosuvastatin.

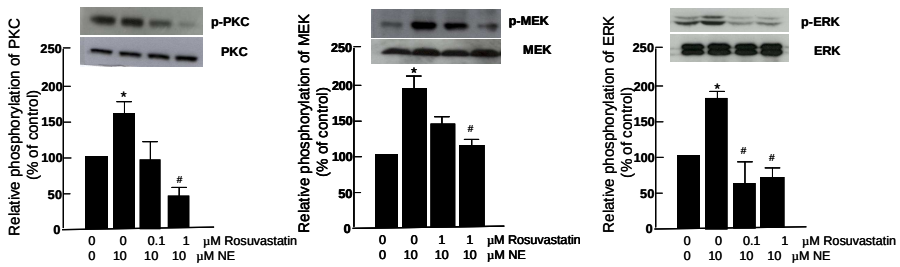

Figure 7. Down-regulation of upstream regulators of ERKs and ERKs by Rosuvastatin. The phosphorylation levels of PKC, MEK, and ERKs were up-regulated by norepinephrine treatment (10 μM, 10min) but decreased by pretreatment with rosuvastatin (1 μM, 12 h). *p<0.05 vs. control, #p <0.05 vs. norepinephrine.

5. Rosuvastatin downregulates proto-oncogene expression in cardiomyocytes.

To determine whether the immediate early genes were influenced by NE we examined the mRNA levels of *c-jun, c-fos,* and *c-myc* in NE-stimulated cardiomyocytes. *c-jun*, *c-fos* and *c-myc* were observed about over 1.5 folds increase after NE (10 μM, 24 h) stimulation. However, only *c-fos* mRNA expression was significantly decreased to the control mRNA level by pretreatment with rosuvastatin (0.1-1 μM, 36 h) (Fig. 8).

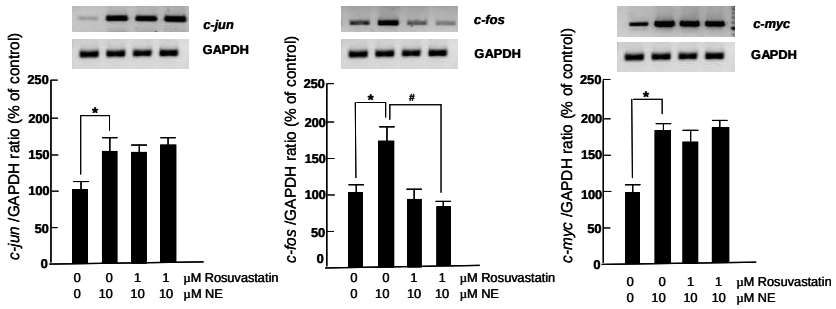

Figure 8. Down-regulation of proto-oncogene expressions by Rosuvastatin. Significant increases in *c-jun*, *c-fos*, and *c-myc* were observed after norepinephrine (10 μM, 24 h) stimulation. However only *c-fos* mRNA expression was significantly decreased by pretreatment with rosuvastatin (0.1-1 μM, 36 h). *$p<0.05$ vs. control, #$p<0.05$ vs. norepinephrine.

6. Rosuvastatin inhibits NE-induced SERCA2a degradation and intracellular Ca^{2+} overload.

The Ca^{2+} ATPase of the sarcoplasmic reticulum (SERCA2) plays a major role in Ca^{2+} homeostasis and contributes to abnormal intracellular Ca^{2+} handling in a failing heart. NE-stimulation induced a significant decrease to about 55% in SERCA2 transcripts after 24 h while pretreatment with rosuvastatin (0.1-1 μM, 36 h) inhibited this decrease in SERCA2 expression (Fig. 9A). The intracellular calcium level was also increased by 2.2 folds in treatment with NE (10 μM, 12 h) and decreased

by pretreatment with rosuvastatin (0.1-1 µM, 24 h) (Fig. 9B).

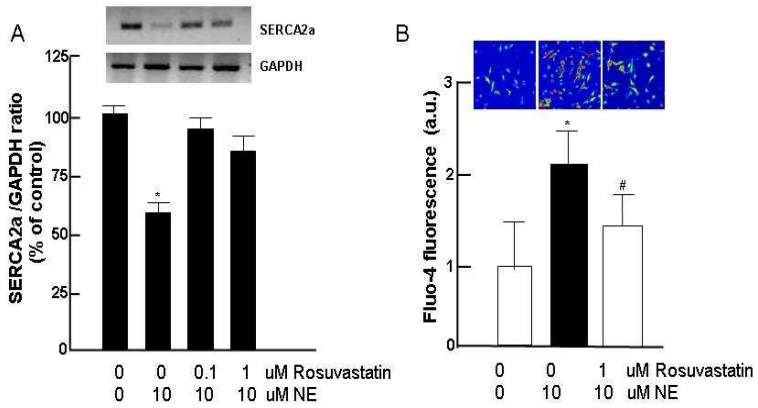

Figure 9. Effect of Rosuvastatin on SERCA2a expression and Ca^{2+} overload in NE-stimulated cardiomyocytes. A. Norepinephrine-stimulation induced a significant decrease in SERCA2a transcripts after 24 h while pretreatment with rosuvastatin (0.1-1 µM, 36 h) inhibited this decrease in SERCA2 expression. B. The intracellular calcium level was also increased by norepinephrine stimulation (10 µM, 12 h) and decreased by pretreatment with rosuvastatin (0.1-1 µM, 24 h). *$p<0.05$ vs. control, #$p<0.05$ vs. norepinephrine.

IV. DISCUSSION

Cardiac hypertrophy is related to an increased risk of cardiac arrhythmias, diastolic dysfunction, congestive heart failure, and death. Cardiac hypertrophy is a compensatory process that occurs in pathological conditions such as hypertension, myocardial infarction, and some genetic heart diseases. Although many studies about hypertrophy in cardiomyocytes have underscored the relationship between $G_{\alpha q}$ and $G_{\alpha 12/13}$, some groups have reported that cardiac hypertrophy with α1-AR stimulation is also related to the G_h pathway [1, 15]. A recent study showed that statins produce favorable effects, such as a cardioprotective effect in hypertensive patients and reverse remodeling in patients with non-ischemic heart failure. However, the exact mechanisms of anti-cardiac hypertrophy and the improvement of cardiac remodeling have not been fully elucidated. Inhibition of isoprenylation of small GTPases has been shown to be a major mechanism of statin induced anti-cardiac hypertrophy. However, few study confirmed the direct inhibitory effects of statin on large G-protein mediated hypertrophy, major pathway of cardiac hypertrophy. Recent study showed that atorvastatin reduced the cAMP- and force-increasing effect of β-adrenergic stimulation in a concentration-dependent

manner in neonatal rat cardiomyocyte. The effect of atorvastatin was accompanied by cytosolic accumulation of a fraction of G-protein γ-subunit with an apparently smaller molecular weight, cytosolic accumulation of G-protein β-subunit, and a decrease in Gαs total protein[24]. In this study, we confirmed the anti-cardiac hypertrophic effects of rosuvastatin via α1-receptor signaling pathway. The α1-receptor signaling plays an important role in the development of cardiac hypertrophy, necrosis and fibrosis, which are often seen with human heart failure and in animal model of NE-induced cardiomyopathy. Especially, in failing human heart, Gh coupled with α1-receptor is an important signal transduction mediator that aggrevates cardiac remodeling[25]. Our results suggest that G_h protein is a novel target of HMG CoA reductase inhibitors in myocardial hypertrophy. Treatment with NE increased G_h expression and membrane translocation. Rosuvastatin inhibited G_h protein activity in cardiomyocytes by inhibiting both basal and NE-stimulated mRNA, protein expression, and membrane translocation. Interestingly, despite treatment with NE, which activated G_q protein and mRNA expressions, treatment with rosuvastatin did not effectively inhibit the NE-stimulated G_q protein and mRNA expression. This finding suggests that the antihypertrophic effects

of rosuvastain were primarily mediated via the α1-AR-Gh-PKC-MEK-ERK pathway. To the best of our knowledge, this is the first report that statins directly suppress G_h-mediated cardiac hypertrophy. In addition to the reversion of phenotypic hypertrophy, rosuvastatin treatment inhibited the NE-induced degradation of SERCA2a and reversed the intracellular calcium overload that plays an important role in the development of heart failure. Rosuvastatin also concentration-dependently inhibited total protein synthesis and downregulated both the basal and NE-induced expression of MLC2v in cardiomyocytes.

The results of our study were consistent with those of recent studies suggesting that atorvastatin and simvastatin inhibit angiotensin II-induced cellular hypertrophy in H9C2 cardiomyoblasts[24, 26,]. Moreover, in this study, we further confirmed that c-fos, proto-oncogene is exclusively suppressed by rosuvastatin, which suggested that c-fos proto-oncogene plays a major role in terms of cardiac hypertrophy. We also found that NE-stimulation of the PKC-MEK1,2-ERKs signaling cascade was inhibited by pretreatment with rosuvastatin. These findings provided us with detailed and specific information about the upstream signaling pathway that was a target of rosuvastatin in the suppression of cardiac hypertrophy. The data we present

provide the evidence in support of using a statin to produce a G_h protein-targeted antihypertrophic effect in the heart.

V. CONCLUSION

In conclusion, we successfully revealed the inhibitory mechanism of rosuvastatin on G_h-protein-mediated cardiac hypertrophy and its upstream regulators. The results of this study provide support for favorable statin effects on patients with heart failure.

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< ABSTRACT(IN KOREAN)>

G-단백질 경로를 통한 3-hydroxy-3-methylglutaryl coenzyme A 환원효소 억제제의 항심비대 효과

<지도교수 정 남 식>

연세대학교 대학원 의학과

최 의 영

최근 3-hydroxy-3-methylglutaryl coenzyme A 환원효소 억제제가 심부전 발생의 주요원인인 심근비대를 억제할 수 있음이 보고되었으며, 그 기전으로 콜레스테롤 생성과정에서 생산되는 GTPase의 생성을 억제함으로써 일어날 수 있다고 연구되었다. 그러나 심근비대를 일으키는 주요 경로인 G-단백질을 통한 스타틴의 심근비대억제 효과에 관해서는 연구된 바가 없다. 이에 본 연구에서는 스타틴이 GTPase와 독립적으로 G-단백질에 작용하여 심근세포의 비대를 억제할 수 있는 지를 연구해 보고자 하였다. 신생백서의 좌심실에서 추출한 심근세포를 대상으로 norepinephrine을 통한 세포비대를 유발하였으며, rosuvastain을 처치하여 세포비대가 억제될 수 있는지와 억제에 관여하는 신호전달 체계를 세포의 총단백/DNA양, 심근세포의 형태학적 크기, 세포내 myosin light chain 의총량, 칼슘

및 칼슘조절인자의 변화를 확인하였으며, 세포신호전달인자에 대한 western blotting과 RT-PCR 기법을 통하여 확인하였다. Norepinephrine 처치를 통하여 α1-adnrenergic 수용체에 선택적으로 작용하여 심근세포의 비대를 유발할 수 있었으며, 이 과정에서 Gh 단백질의 활성과 세포막에서의 Gh 단백질에 의한 신호전달작용을 확인할 수 있었으며 PKC-MEK-ERK 전달체계의 활성화를 확인할 수 있었다. 이후 Rosuvastain 을 처치하여 심근세포의 norepinephrine에 의한 비대를 억제할 수 있었으며, 세포막에서의 선택적으로 Gh 단백질의 활성을 억제할 수 있었다. Rosuvastatin 은 용량에 비례하여 norepinephrine으로 인해 증가된 세포내 총단백질/DNA 용량, myosin light chain-2의 양, 세포표면적을 감소시킬 수 있었으며, 세포전달인자인 PKC, MEK1,2, ERK 의 인산화를 억제시킬 수 있었다. 또한 Rosuvastain 을 처치하여 norepinephrine으로 인해 발생한 세포내 SERCA2a의 비활성화 및 칼슘의 과부하를 조절할 수 있었다. 결론적으로 rosuvastatin은 Gh 단백질에 직접적으로 작용하여 α1-adrenergic 수용체를 통한 심근세포 비대를 억제할 수 있음과 관련된 신호전달체계를 증명할 수 있었다.

핵심되는 말 : 3-hydroxy-3-methylglutaryl coenzyme A 환원효소, 심근세포비대