Hydroxymethylglutaryl Coenzyme A Reductase Inhibitor Simvastatin Prevents Cardiac Hypertrophy Induced by Pressure Overload and Inhibits p21ras Activation

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Background—Patients with cardiac hypertrophy are at increased cardiovascular risk. It has been hypothesized that hydroxymethylglutaryl coenzyme A reductase inhibitors may exert beneficial effects other than their cholesterol-lowering actions. The aims of the study were to assess the in vivo effects of simvastatin (SIM) on cardiac hypertrophy and on Ras signaling in rats with ascending aorta banding.

Methods and Results—Wistar rats were randomized to receive either treatment with SIM or placebo, and then short-term (group I) and long-term (group II) left ventricular pressure overload was performed by placing a tantalum clip on ascending aorta. At the end of treatment period, left and right ventricular weight, body weight, and tibial length were measured and echocardiographic evaluations were performed. Ras signaling was investigated by analyzing Ras membrane localization and activation, ERK2 phosphorylation, and p27 kip1 and cdk4 levels. In SIM-treated rats, a significant reduction of left ventricular weight/body weight, echocardiographic left ventricular mass, and left ventricular end-diastolic diameter and end-diastolic pressure was found. In rats with pressure overload, SIM treatment significantly reduced Ras membrane targeting, Ras in vivo activation, ERK2 phosphorylation, and the ratio cdk4/p27 kip1.

Conclusions—HMG CoA inhibitor SIM inhibits in vivo Ras signaling and prevents left ventricular hypertrophy development in aortic-banded animals. (Circulation. 2002;106:2118-2124.)

Key Words: hypertrophy | signal transduction | statins | myocardium | pressure

Clinical trials indicate that cardiac hypertrophy is a major risk factor for the development of congestive heart failure and death. Although hypertrophy is a compensatory response to increased wall stress in its earliest stages, it has been demonstrated recently in mice that development of pressure-overload cardiac hypertrophy and normalization of wall stress does not prevent left ventricular (LV) decompensation, as it was previously hypothesized. In fact, it is associated with progressive deterioration in cardiac function and LV chamber enlargement.

Ventricular hypertrophy is associated with changes in myocyte morphology, increased cell size and myofibrillogenesis, and changes in the pattern of gene expression. Various signal transduction pathways have been involved in the development of hypertrophy. Ras-Raf1-ERK1 kinase cascade is the main signaling system activated in various models of hypertrophy. Transgenic mice expressing HaRas, specifically in the heart, display the hallmarks of LV hypertrophy (LVH). A recent study has shown that transgenic mouse lines with cardiac-restricted expression of active MEK1 develop cardiac hypertrophy and a dramatic increase in cardiac function.

Plasma membrane localization of GTP-binding proteins such as Ras and RhoA is crucial for their biological activity. In fact, their prenylation is dependent on the formation of mevalonate-derived isoprenoid compounds as the farnesyl-pyrophosphate and geranyl-geranyl-pyro-phosphate. It has been previously demonstrated that selective inhibition of farnesyl protein transferase blocks Ras processing and that the inhibition of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase interferes with Ras anchorage to cell membrane and its activation by reducing the pool of farnesyl groups.

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Clinical trials indicate that HMG-CoA reductase inhibitors greatly reduce cardiovascular-related morbidity and mortality in patients with and without coronary heart disease.12–15 The mechanisms of these beneficial effects are still not completely known. Statins may exert biological effects other than their cholesterol-lowering actions,16–18 including inhibition of Ras activation by preventing isoprenylation.11 A recent study has shown that lipid-soluble HMG-CoA reductase inhibitors prevent angiogenesis II-induced hypertrophy at least in part through inhibition of p21ras/mitogen-activated protein kinase A pathway in neonatal cardiac myocytes in vitro18 and that simvastatin (SIM) induces regression of cardiac hypertrophy and fibrosis and improves cardiac function in a transgenic rabbit model of human hypertrophic cardiomyopathy.19 Presently, in vivo effects of HMG-CoA reductase inhibitors on Ras signaling and on downstream cell-cycle regulators in cardiac hypertrophy induced by pressure overload are still not well defined. Accordingly, the aims of the present study were to specifically assess the effects of SIM on Ras signaling in vivo and on cardiac hypertrophy development after ascending aortic banding.

Methods
Experimental In Vivo Study Protocol
The animals in this study were handled according to the animal use principles of the American Society of Physiology. To test the effects of SIM on LV hypertrophy in vivo, two different protocols were used.

Group I: Short-Term Pressure Overload
In rats in group I, the effects of SIM pretreatment on LV hypertrophy induced by acute pressure overload were tested. In male adult Wistar rats (180 to 200 g; age 9 to 11 weeks; n = 66) (Morini, S. Polo d’Enza, Italy) anesthetized with an intramuscular injection of ketamine 100 mg/kg and xylazine 5 mg/kg, aortic stenosis was created as previously described20 by use of tantalum clip (Weck Inc) of 0.58-mm internal diameter placed on the ascending aorta via a thoracic incision (LVH). Additional age-matched controls underwent a left thoracotomy without placement of the clip (SHAM). In group I, rats were randomized to treatment with SIM (Merck Sharp and Dohme) 40 mg/kg per day added to drinking water or placebo for 15 days, then aortic banding was performed and drug or placebo treatment continued for 10 days. The study cohort of group I consisted of the following 3 arms: SIM-treated rats with aortic stenosis (LVH SIM-I; n = 24), untreated rats with aortic stenosis (LVH-I; n = 21), and sham-operated animals (SHAM-I; n = 21).

Group II: Long-Term Pressure Overload
In rats in group II, the effects of SIM chronic treatment on LV hypertrophy and transition to heart failure induced by chronic pressure overload were tested. In male young Wistar rats (60 to 70 g; age 3 to 4 weeks; N = 30), aortic stenosis was established (LVH) as indicated above. Additional age-matched animals underwent a left thoracotomy without placement of the clip (SHAM). In group II, 6 weeks after aortic banding, rats were randomized to receive either treatment with SIM 40 mg/kg per day added to drinking water or no treatment. Drug or no drug treatment continued for 15 weeks (weeks 6 to 21 after banding). The study cohort of group II consisted of the following 3 arms: SIM-treated rats with aortic stenosis (LVH SIM-II; n = 11), untreated rats with aortic stenosis (LVH-II; n = 10), and sham-operated animals (SHAM-II; n = 9).

At the end of the 10-day (in group I) and 15-week (in group II) treatment periods, rats were anesthetized and, after calculation of body weight (BW) and tibial length (TL), in vivo LV end-systolic pressure (LVEPS) and LV end-diastolic pressure (LVEDP) were measured and the hearts were removed to measure heart weight, LV weight (LVW), and right ventricular weight. Only rats with LVEPS between 180 and 200 mm Hg were included in the study.

Serial Echocardiographic Assessment
In rats in group II (long-term ascending aortic banding), serial echocardiograms were performed with a 7.5-MHz phased-array transducer, as previously reported.21

Toxicity
To study SIM toxicity, laboratory studies were performed at baseline and at the end of treatment period in group II animals.

Total Protein Extracts
Hearts from rats of group I were homogenized in ice-cold radio immunoprecipitation assay buffer using a homogenizer. The lysate was extracted on ice for 20 minutes and then was centrifuged at 10 000g for 30 minutes at 4°C. The supernatant was transferred to a new tube, and total protein concentrations were determined.

Membrane/Cytosol Fractions
Hearts from rats of group I 10 days after aortic banding were homogenized in ice-cold sucrose buffer using a homogenizer. The lysates were extracted on ice and were centrifuged; the pellets were excluded and the supernatants were then centrifuged. The supernatants (Cytosol) were transferred to a new tube, and the pellets (membranes) were resuspended in RIPA buffer.

Immunoblotting
Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes that were incubated with specific antibodies and then with goat IgG conjugated to horseradish peroxidase; bands were visualized by enhanced chemiluminescence (Amersham Life Sciences Inc) according to the manufacturer’s instructions and quantified using densitometry. Measurements of p27kip1, cdk4 proteins were performed on protein extracts of hearts of group I 6 days after aortic banding; other analysis were performed on hearts 10 days after surgery. Each experiment and densitometric quantification was separately repeated at least 4 times.

Ras Activation Assay
In vivo Ras activation was tested as previously described.22 Briefly, hearts from rats of group I, 10 days after surgery, were lysed and then centrifuged. Supernatants were collected, and protein concentrations were determined. A total of 100 μg of protein was collected from each group and stored as an input for the affinity precipitation reactions. At least 10 μg of protein extracts were used for affinity precipitation. Lysates were incubated with GST-Raf1 prebound to glutathione Sepharose for 1 hour at 4°C rocking. Bound proteins were eluted with SDS-PAGE sample buffer, resolved on 15% acrylamide gels, and subjected to immunoblotting. Blots were probed with anti-pan Ras antibodies (Santa Cruz).

Statistical Analysis
All values are expressed as mean±SEM. Statistical analysis of differences observed between groups was done by ANOVA and unpaired t test using SPSS 10.0 software. The Tukey’s test was applied to compare single mean values. A linear regression analysis was used to compare the measurement of LV mass obtained with postmortem LV weight and in vivo echocardiography. P<0.05 was considered significant.

Results
Statins Reduce Cardiac Hypertrophy Induced by Short- and Long-Term Pressure Overload
Aortic banding leads to cardiac hypertrophy in vivo. Short (group I, 10 days) or long (group II, 15 weeks) banding periods are sufficient to induce cardiac hypertrophy, as shown
in Table 1 and Figure 1. Specifically, animals in both groups show a significant increase in LV weight compared with the controls (SHAM).

Pretreatment with SIM resulted in a significant inhibition of LV hypertrophy in group I (∼25% reduction of LVW, 32% reduction in LVW/BW, and 30% reduction in LVW/TL compared with LVH rats) and in group II (∼30% reduction of LVW, 30% reduction in LVW/BW, and 27% reduction in LVW/TL compared with LVH rats), as shown in Figure 1 and Table 1. In both groups, no significant differences were observed in BW, TL, and right ventricular weight.

**Echocardiography and LV Pressures**

In vivo echocardiographic measurements were obtained in all rats included in group II (Table 2). At baseline, no significant differences in LV diastolic cavity dimensions were observed among groups (data not shown). LV mass was significantly increased in aortic banded rats. At the end of the 15-week treatment period, a significant reduction in LV mass (0.70 ± 0.05 versus 0.96 ± 0.06 g; P < 0.05) and end-diastolic diameter (6.53 ± 0.22 mm versus 7.17 ± 0.15 mm; P < 0.05) was found in rats treated with SIM compared with LVH rats.

The LV pressures are reported in Figure 2. In both groups, LV systolic pressure was increased after aortic stenosis and was not affected by the treatment. Interestingly, in vivo LVEDP was significantly lower in SIM-treated rats. No significant differences in systemic blood pressure and heart rate were observed in the groups of animals studied (data not shown).

**Statins Inhibit Ras-ERK1/2 Signaling**

Aortic banding significantly stimulated Ras-ERK1/2 signaling, as shown by increased Ras activation (Figure 4), ERK1/2 phosphorylation (Figure 5), and high cdk4/p27 ratio (Figure 6). ERK1/2, p27, and cdk4 are downstream effectors of Ras-Raf1 cascade. Ras is another GTP-binding protein involved in the development of cardiac hypertrophy, and prenylation and phosphorylation is crucial even for its plasma membrane localization and biological activity. To address in vivo effects of SIM on Ras signaling, we analyzed by immunoblot in hearts 10 days after aortic banding Ras and RhoA membrane targeting, Ras in vivo activation, ERK1/2 phosphorylation, and cdk4/p27 ratio. We also evaluated ERK2 and the glucose transporter, GLUT1 protein levels, in membrane/cytosol fractions to ensure the good quality of the preparation and to demonstrate that differences observed between groups were not attributable to different protein concentrations.

Total Ras, RhoA, and ERK2 protein levels did not change when SHAM-, LVH-, and SIM-treated animals were compared (Figure 3). We consistently found that GLUT1 levels were slightly stimulated in LVH animals. Note that the higher levels of GLUT1 were not sensitive to SIM treatment (Figure 3). To analyze the fraction of Ras and RhoA proteins bound to the membranes, we fractionated total heart proteins (Figure 3). As expected, Ras and RhoA proteins were predominantly membrane-bound in all groups of animals. In hearts derived from SIM-treated hypertrophic rats (LVH-SIM), Ras proteins levels in membrane fractions were significantly lower compared with their hypertrophic nontreated controls (LVH) and LVH-SIM.
to SHAM-operated animals. A similar pattern of intracellular partitioning was observed with RhoA proteins (Figure 3). Thus, inhibition of isoprenoid synthesis by SIM prevented Ras and RhoA membrane localization and determined a significant reduction in their membrane levels. We did not find accumulation of Ras and RhoA proteins in the cytosol in SIM-treated animals. Under these conditions, Ras and RhoA in the cytosol might have a shorter half life compared with the membrane-bound forms.

Statins did not influence the partition or the amount of other membrane-bound proteins, such as the glucose transporter (GLUT1), or other signaling molecules, such as ERK1/2 (Figure 3). Thus, ERK2 was localized predominantly in the cytosol, without any significant difference in protein levels between different groups of animals. GLUT1 proteins were detectable in membrane fractions (with nearly undetectable levels in the cytosol), and SIM treatment did not change GLUT1 membrane localization (Figure 3).

To determine if Ras activation was impaired in LVH- or SIM-treated animals, we developed an affinity assay exploiting the known specificity of the interaction between active Ras and Raf1 (Figure 4). A significant Ras activation was found in LVH animals 10 days after aortic banding, when cardiac hypertrophy was clearly measurable. More interestingly, Ras activation was completely prevented by SIM administration in vivo. We also investigated RhoA, cdc42, and rac activation during aortic banding and were not able to resume conclusive data on Rho family protein activation (data not shown).

Moreover, we investigated total and phosphorylated ERK2, which represents the active enzyme that mediates downstream Ras signals. Absolute levels of ERK2 did not change in SHAM-, LVH-, and SIM-treated animals (Figure 5). Immunoblotting of the same gel with anti-pERK2 antibodies showed a consistent 2-fold increase in the activated enzyme levels in LVH relative to SHAM and a significant reduction in SIM-treated, aortic-banded rats (Figure 4). ERK phosphorylation in sham-operated animals is variable, although it never reaches statistical significance compared with nonoperated animals (data not shown).

Previous studies demonstrated that overexpression of dominant-negative forms of Ras completely blocked PDGF-induced p27kip1 degradation, that the Ras/Raf pathway is important for the induction of cyclin D/CDK4/CDK6 activity,23,24 and that RhoA regulates p27kip1 degradation through its activation of cyclin E/CDK2 activity. The ratio of cdk4 to p27 is a critical factor that determines entry in the cycle and growth.28–30 In hearts from LVH rats, 6 days after aortic banding, there was a significant increase of cdk4 levels associated with a significant reduction of p27kip1 levels compared with SHAM animals (Figure 5). Reduction of the cdk inhibitor p27 and the parallel increase of cdk4 indicate that Ras signaling is active. SIM completely reversed the increase of cdk4 and the reduction of p27kip1 levels in hearts from aortic-banded rats (Figure 5). As a consequence, the cdk4/p27kip1 ratio in the hearts of LVH animals was significantly lower (Figure 5). Finally, as expected, compared with LVH and SHAM groups (respectively, plasma cholesterol of 94.3±7.3 and 99.7±8.5 mg/dL), SIM administration reduced plasma cholesterol in the LVH-SIM group (47.8±6.4; P<0.05 versus LVH and SHAM) but was not toxic under our experimental conditions, as shown by the absence of statistically significant differences both in hepatic functions (aspartate aminotransferase [AST], 107±13 IU/L and alanine aminotransferase [ALT], 39±5 IU/L in LVH-SIM group) and in skeletal muscle lysis (creatine kinase [CK], 428±29 IU/L in LVH-SIM group) compared with SHAM and LVH groups (GOT, 101±11 IU/L; GPT, 35±7 IU/L; CK, 415±31 IU/L and GOT, 111±12 IU/L; GPT, 37±6 IU/L; CK, 432±27 IU/L, respectively; P=NS versus LVH-SIM).

### Discussion

The major findings of the present study are that statin administration to rats subjected to LV pressure overload by

### TABLE 2. Echocardiographic Data From Rats of Group II at the End of 15-Week Treatment Period

<table>
<thead>
<tr>
<th></th>
<th>EDD, mm</th>
<th>ESD, mm</th>
<th>FS, %</th>
<th>PWs, mm</th>
<th>PWD, mm</th>
<th>ILS, mm</th>
<th>ISd, mm</th>
<th>LVM, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>6.62±0.12</td>
<td>3.71±0.14</td>
<td>43.6±2.8</td>
<td>2.41±0.18</td>
<td>1.37±0.11</td>
<td>2.36±0.19</td>
<td>1.42±0.08</td>
<td>0.57±0.05</td>
</tr>
<tr>
<td>LVH</td>
<td>7.17±0.15*</td>
<td>3.96±0.15</td>
<td>44.7±1.4</td>
<td>2.92±0.19</td>
<td>1.89±0.08*</td>
<td>2.56±0.20</td>
<td>1.81±0.10*</td>
<td>0.96±0.06*</td>
</tr>
<tr>
<td>SIM</td>
<td>6.53±0.22</td>
<td>3.77±0.18</td>
<td>42.4±1.2</td>
<td>2.71±0.10</td>
<td>1.66±0.07†</td>
<td>2.49±0.09</td>
<td>1.60±0.08†</td>
<td>0.70±0.05†</td>
</tr>
</tbody>
</table>

EDD indicates end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; PWs, systolic posterior wall dimension; PWD, diastolic posterior wall dimension; ILS, systolic interventricular septum dimension; ISd, diastolic interventricular septum dimension; LVM, left ventricular mass; SHAM, sham-operated animals; LVH, aortic-banded rats; and SIM, SIM-treated, aortic-banded rats.

*P<0.05 vs SHAM and LVH-SIM.
†P<0.05 versus SHAM.

![Figure 2](http://circ.ahajournals.org/)

Figure 2. Bar graphs showing in vivo LVESP and LVEDP measured in intact animals at the end of study in groups I and II. Treatment with SIM significantly reduced LVESP but did not change the LVEDP. *P<0.0001 for SHAM vs LVH and SIM; §P<0.01 for SHAM vs LVH and SIM; #P<0.01 for SIM vs LVH. SHAM indicates sham-operated animals; LVH, untreated aortic-banded rats; SIM, SIM-treated aortic-banded rats.
aortic banding prevents cardiac hypertrophy, inhibits Ras membrane targeting and activation, inhibits ERK1/2 activation, and lowers cdk4/p27kip1 ratio. The antihypertrophic effects of chronic SIM administration occur in the presence of an expected elevation of LV systolic pressure caused by surgical clipping of ascending aorta, indicating that statins may favorably modify the development of hypertrophy without affecting loading conditions.

Cardiac hypertrophy is an important adaptive response. In both humans and animal models, pressure overload is characterized by a period of compensation in which concentric hypertrophic remodeling normalizes systolic wall stress and other parameters of contractility. This period of adaptation, which lasts for weeks in rodents and months to years in humans, inexorably leads to heart failure. Recent studies demonstrate that the degree of increased LV muscle mass is an effective and independent risk factor for cardiac mortality and that regression of cardiac hypertrophy is associated with a decreased risk of cardiovascular disease.

The ascending aortic banding model of LV hypertrophy is an ideal and a better model to assess myocardial response to pressure overload and, in addition, to evaluate possible therapeutic strategies. In fact, this model excludes the activation of the renin-angiotensin system and modification of norepinephrine levels and systemic pressure.

Over the last decade, Ras-Raf1-ERK1 signaling has been extensively studied in the heart, and its role in cardiac hypertrophy development is now widely accepted. Indeed, transgenic mouse lines with cardiac-restricted expression of active MEK1 develop cardiac hypertrophy and a dramatic increase in cardiac function. Our data confirm and extend these results. We find a significant Ras/ERK1/2 activation and a reduction of the ratio cdk4/p27 after aortic banding (Figures 4, 5, and 6). Although ERK1/2 might be activated by other pathways, the inhibition of ERK1/2 and prevention of Ras activation, after treatment with statins (Figures 4 and 5), indicates that Ras inhibition is the primary cause of the downregulation of ERK1/2 cascade. In fact, inhibition of Ras membrane targeting inhibits its action and consequently the activation of the cell-cycle kinase cdk4 and degradation of the negative cell-cycle regulator CDKI-p27, but in our experimental model we cannot resume conclusive data on Ras membrane targeting in aortic-banded rats. Recent data indicate that statins inhibit angiotensin II–induced cardiac myocyte hypertrophy and RhoA activation in vitro, but in our experimental model we cannot resume conclusive data on RhoA activation in vivo during pressure overload in the rat.

The present study demonstrates that HMG-CoA reductase inhibitor, SIM, is able to affect the degree of LV hypertrophy and to slow the transition to heart failure. In fact, both short-term aortic-banded treated rats (group I) and long-term aortic-banded treated rats (group II) showed a significant reduction of LV mass (Figure 1). Also, administration of SIM 6 weeks after aortic banding (group II) reduced the degree of LV hypertrophy and slowed down the transition to LV dilatation. At the end of the 15-week treatment period, in fact, treated rats in group II showed, with reduction of LV mass, a significant reduction of LV end-diastolic diameter. These effects were associated, in both groups, with a significant reduction of LV end-diastolic pressure and that reduction of cardiac hypertrophy is associated with a decreased risk of cardiovascular disease.

Although the statin dose used in this study is high compared with that used in clinical setting, we did not find any evidence of adverse or toxic effects in short- and long-term treatments. In addition, previous studies demonstrated species-specific differences in SIM metabolism; in fact the DL-3a of this drug in rats is 5 g/kg per day (Merck Sharp and Dhome database), and other animal studies have shown significant biological effect using doses ranging from 10 to 150 mg/kg per day. In treated animals, a reduction of cholesterol levels was found, but in sham animals treated with SIM, no significant modification of cardiac mass was ob-
served (data not shown). It is unclear whether cholesterol levels may play a role in hypertrophy induced by aortic banding in our model. Finally, although the number of rats included in the study was not sufficient to reach a statistical power to evaluate survival, we observed a reduction of mortality rate in rats treated with SIM (14% versus 38% of control-banded rats). Additional prospective studies should be performed to evaluate the effects of SIM on survival rate in a large number of animals over a period of at least 12 months after aortic banding or myocardial infarction.

In conclusion, the data presented in this study document a novel effect of HMG CoA reductase inhibitors on LV hypertrophy induced by aortic banding in rats and open a new therapeutical strategy for patients with cardiac hypertrophy. These data could be of great relevance in designing future clinical trials to test new therapeutical strategies in patients at risk of development of cardiac hypertrophy.

References

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