Rapamycin Attenuates Load-Induced Cardiac Hypertrophy in Mice

Tetsuo Shioi, MD, PhD; Julie R. McMullen, PhD; Oleg Tarnavski, MD; Kimber Converso, RDMS; Megan C. Sherwood, MBBS, FRACP; Warren J. Manning, MD; Seigo Izumo, MD

Background—Cardiac hypertrophy, or an increase in heart size, is an important risk factor for cardiac morbidity and mortality. The mammalian target of rapamycin (mTOR) is a component of the insulin–phosphoinositide 3-kinase pathway, which is known to play a critical role in the determination of cell, organ, and body size.

Methods and Results—To examine the role of mTOR in load-induced cardiac hypertrophy, we administered rapamycin, a specific inhibitor of mTOR, to mice with ascending aortic constriction. Activity of p70 ribosomal S6 kinase 1 (S6K1), an effector of mTOR, was increased by 3.8-fold in the aortic-constricted heart. Pretreatment of mice with 2 mg · kg$^{-1}$ · d$^{-1}$ of rapamycin completely suppressed S6K1 activation and S6 phosphorylation in response to pressure overload. The heart weight/tibial length ratio of vehicle-treated aortic-banded mice was increased by 34.4±3.6% compared with vehicle-treated sham-operated mice. Rapamycin suppressed the load-induced increase in heart weight by 67%. Attenuation of cardiac hypertrophy by rapamycin was associated with attenuation of the increase in myocyte cell size induced by aortic constriction. Rapamycin did not cause loss of body weight, lethality, or left ventricular dysfunction.

Conclusions—mTOR or its target(s) seems to play an important role in load-induced cardiac hypertrophy. Because systemic administration of rapamycin has been used successfully for the treatment of transplant rejection in clinical practice, it may be a useful therapeutic modality to suppress cardiac hypertrophy in patients. (Circulation. 2003;107:1664-1670.)

Key Words: hypertrophy ■ heart failure ■ signal transduction

The insulin–phosphoinositide 3-kinase (PI3K) pathway plays a critical role in the determination of cell, organ, and body size in Drosophila,$^{1}$ and PI3K(p110α) and Akt (a downstream effector of PI3K) promote heart growth in transgenic mice.$^{2,3}$ The target of rapamycin (TOR) has been shown to interact genetically with the PI3K pathway in Drosophila.$^{4,5}$ Rapamycin is a lipophilic macrolide, which was first identified in a natural product screen as a fungicide isolated from the soil bacterium Streptomyces hygroscopicus.$^{6}$ In the search for molecules affected by rapamycin, TOR was identified in yeast (reviewed by Gingras et al$^{7}$). Purification and molecular cloning of mammalian TOR (mTOR) revealed a 290-kDa protein that is closely related to yeast TOR. Rapamycin has effects on growth by forming a gain-of-function inhibitory complex with FKBP12 (FK506-binding protein; MW, 12 kDa)$^{8,9}$ This complex binds to mTOR and inhibits its function.

In response to growth factors and amino acids, mTOR controls the mammalian translational machinery via activation of p70 ribosomal S6 kinase and via inhibition of the eIF4E inhibitor, 4E-BP1.$^{8}$ p70 ribosomal S6 kinase is a physiological kinase for the 40S ribosomal S6 protein.$^{8,9}$ In mammals, 2 highly homologous genes, called p70 ribosomal S6 kinase 1 (S6K1) and p70 ribosomal S6 kinase 2 (S6K2), have been identified.$^{10}$ Rapamycin is a potent inhibitor of S6K1, preventing activation of S6K1 by all known agonists.$^{11}$ Although S6K1 is not the direct target of rapamycin, it is likely to be a proximal effector, because inactivation of S6K1 by rapamycin occurs within 5 minutes. The inhibitory effects of rapamycin are considered to be highly specific, because the kinase most closely related to S6K, the 90-kDa ribosomal S6 kinase (RSK), is resistant to the drug.$^{11}$

Rapamycin effectively attenuated hypertrophy of cultured myocytes induced by several growth factors.$^{12,13}$ Furthermore, S6K1 was activated in a cat model of right ventricular pressure overload, suggesting that mTOR or its target(s) might be involved in load-induced cardiac hypertrophy.$^{14}$ To determine the role of mTOR or its target(s) in load-induced cardiac hypertrophy, we administered a clinically relevant dose of rapamycin to mice subjected to ascending aortic constriction.
Methods

Generation of Mice With Aortic Stenosis

For aortic-banding experiments, ascending aortic constriction was performed in 12-week-old male FVB/N mice (Charles River Laboratories International, Inc, Wilmington, Mass) as described previously, except that mice were anesthetized with pentobarbital sodium salt (60 mg/kg) and the aorta was ligated with a 25-gauge needle. For the sham operation, the aorta was not ligated.

Administration of Rapamycin

Rapamycin (2 mg · kg−1 · d−1) or vehicle was administered intraperitoneally to aortic-banded or sham-operated mice. The dose of rapamycin was based on the literature in which rapamycin was used as an immunosuppressant in mice. A dose of 2 mg · kg−1 · d−1 in mice is equivalent to a dose of 0.17 mg · kg−1 · d−1 in humans when normalized by body surface area. This dose is comparable to the dose of rapamycin used in human studies. The solvent for rapamycin was 0.2% sodium carboxymethylcellulose, 0.25% polysorbate-80 in water. Vehicle was the solvent for rapamycin.

Experimental Protocols

Protocol 1

To examine whether acute pressure overload induces S6K1 activation and S6 phosphorylation, aortic-banded or sham-operated mice were killed 1, 4, 8, 24, and 48 hours and 1 week after surgery.

Protocol 2

To examine the effect of rapamycin on S6K1 activity, S6 phosphorylation, e-Jun NH2-terminal kinase 1 (JNK1) activity, and glycogen synthase kinase-3β (GSK3β) phosphorylation, rapamycin- or vehicle-treated mice were subjected to the sham or banding operation and killed 4 hours after surgery. Rapamycin or vehicle was given on the day before surgery and 3 hours before surgery.

Protocol 3

To examine the effect of rapamycin on load-induced cardiac hypertrophy, we treated aortic-banded and sham-operated mice with rapamycin or vehicle for 1 week after surgery. Rapamycin or vehicle treatment was started on the day before the operation, injected 3 hours before surgery, and then injected daily for 1 week. Survival was monitored, echocardiography was performed 6 days after surgery, and mice were killed 7 days after surgery. Body weight, heart weight, lung weight, and tibial length were measured. Heart tissues were used for analysis of cell size and gene expression.

Protocol 4

To assess hemodynamic loading conditions, left ventricular (LV) pressures were measured in sham or banded mice after receiving vehicle or rapamycin for 1 week (as described in protocol 3). Mice were then killed, and heart weight and tibial length were measured.

Echocardiography

Echocardiography was performed as described previously, except that 2,2,2-tribromoethanol (Aldrich; 0.4 to 0.6 mg/kg) was used for anesthesia.

Hemodynamic Measurements

Mice were anesthetized and ventilated as described for the aortic banding operation. LV pressures were measured by a 1.4F high-fidelity pressure catheter (Millar catheter) inserted through the LV apex. The catheter was secured in the LV apex with a purse-string suture using 5-0 silk. Pressure signals were recorded at 2 kHz for 5 minutes, stored to disk, and analyzed with PowerLab software (Chart 4.1.2, ADInstruments).

Measurement of S6K1 Activity in Heart Tissue

Heart lysates were obtained and S6K1 activity was measured as described previously.

Rapamycin Attenuates Cardiac Hypertrophy

Heart lysates (100 µg) were subjected to SDS-PAGE as described previously. To measure S6 phosphorylation, the blots were probed with an anti–phospho S6 antibody (New England Biolabs) followed by an anti-GAPDH antibody (Research Diagnostics). To measure the phosphorylation of GSK3β, the blots were probed with an anti–phospho GSK3β antibody (Cell Signaling) followed by an anti-GSK3β antibody (Transduction Laboratory).

Measurement of JNK1 Activity in the Heart

The activity of JNK1 was measured by immune complex kinase assays as described previously.

Myocyte Cross-Sectional Area

Hearts were cut at the horizontal short-axis plane, fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Heart sections cut at the level of the papillary muscle were selected and used for the measurement of cross-sectional area. The sections were deparaffinized, rehydrated, and incubated for 1 hour at room temperature with FITC-labeled wheat germ agglutinin (Sigma; 1:5 dilution) to visualize myocyte membranes. Images of LV cardiomyocyte cell membranes were obtained with a confocal microscope (Bio-Rad). Regions that included the circular shape of capillaries were selected from the epicardial side of the LV free walls. Morphometric analysis was performed with IPLab software (Scana-lytics, Inc). Mean values from each mouse were calculated by use of the measurements from 60 to 80 cells from an individual mouse. Next, new mean values (±SEM) for each experimental group were calculated on the basis of the data from the mean values from individual mice and are presented.

Northern Hybridization

Northern hybridization was performed as described previously. Mouse atrial natriuretic peptide (ANP) cDNA and mouse brain natriuretic peptide (BNP) cDNA were used.

Statistical Analysis

All values are expressed as mean±SEM. Differences between the groups were compared by 2-way ANOVA followed by the Fisher’s protected least significant difference post hoc test. A value of P<0.05 was considered significant.

Results

Effect of Acute Pressure Overload on S6K1 Activity and S6 Phosphorylation

First, we examined whether S6K1, a target of mTOR, was activated in response to acute pressure overload (protocol 1). Aortic-banded or sham-operated mice were killed 1, 4, 8, 24, and 48 hours and 1 week after the operation (n=4 for each time point). At 1, 4, and 8 hours after the operation, S6K1 activity in the hearts from sham-operated mice was decreased compared with that from mice that had not undergone surgery (unoperated mice) (Figure 1A). In contrast, S6K1 activity was increased 1 hour after aortic banding, reached its peak at 4 hours, and was similar to baseline levels at 24 hours (Figure 1A).

To confirm the activation of S6K1 in heart tissue in response to aortic banding, phosphorylation of S6 was measured (Figure 1B). As seen with S6K1 activity, the amount of phosphorylated S6 in the hearts from sham-operated mice was decreased after the operation compared with unoperated mice. The amount of phosphorylated S6 was markedly increased 4 hours after aortic banding compared with sham-operated animals. Thus, acute pressure overload induced S6K1 activation and S6 phosphorylation in the heart.
Effect of Rapamycin on S6K1 Activity, S6 Phosphorylation, JNK1 Activity, and GSK3β Phosphorylation

Next, we examined the effects of rapamycin on load-induced S6K1 activity (protocol 2). Because S6K1 activity was highest 4 hours after the banding operation, we examined S6K1 activity in the hearts from rapamycin-treated mice at this time point (Figure 2A). Rapamycin (2 mg · kg⁻¹ · d⁻¹) suppressed basal S6K1 activity as well as that in response to acute pressure overload. Rapamycin completely inhibited basal activity as well as the load-induced increase in S6 phosphorylation (Figure 2B).

We also examined the effect of rapamycin on other signaling molecules in response to aortic banding. Mitogen-activated protein (MAP) kinases are postulated to be mediators of cardiac hypertrophy.23 We examined the activity of extracellular signal–regulated kinase 2 (ERK2), p38 MAP kinase, and JNK1 in aortic-banded hearts 4 hours after the operation. JNK1 was activated 5.6±0.4-fold in vehicle-treated aortic-banded mice compared with vehicle-treated
sham-operated mice (Figure 2C). ERK2 or p38 MAP kinase was not significantly activated in the aortic-banded hearts at this time point (data not shown). JNK1 activity was partially but significantly reduced in rapamycin-treated aortic-banded mice compared with vehicle-treated aortic-banded mice. We also examined the phosphorylation of GSK3β, a well-defined direct target of Akt that has been shown to negatively regulate heart growth.24,25 In the present study, phosphorylation of GSK3β was not different between aortic-banded and sham-operated mice. Furthermore, rapamycin treatment did not affect the phosphorylation of GSK3β (Figure 2D).

Effect of Rapamycin on the Load-Induced Increase in Heart Weight

To examine whether rapamycin attenuates load-induced cardiac hypertrophy, we treated aortic-banded mice with rapamycin or vehicle daily for 1 week after surgery (protocol 3). There was no significant difference in mortality between the groups (0 of 6 vehicle-treated sham, 4 of 12 vehicle-treated banded, 1 of 6 rapamycin-treated sham, and 2 of 12 rapamycin-treated banded died within 1 week of the operation). Mice were killed 1 week after the operation. The heart weight/tibial length ratio of aortic-banded mice was increased by 34.4±3.6% compared with vehicle-treated sham-operated mice (Table 1). In contrast, the heart weight/tibial length ratio of rapamycin-treated banded mice was increased by only 11.5±4.4% compared with rapamycin-treated sham mice. Thus, rapamycin suppressed the load-induced increase in heart weight/tibial length ratio by 67%. Rapamycin did not affect body weight, lung weight, or liver weight (Table 1).

Effect of Rapamycin on Hemodynamic Loading Conditions

To examine the effect of rapamycin on hemodynamic loading conditions of aortic-banded mice, we measured LV pressures (protocol 4). The peak systolic pressures were relatively low in the present study (Table 2). This can probably be explained by the effects of anesthesia and the invasive technique used to measure LV pressures in open-chest mice. Because aortic-banded mice display signs of pressure overload, including labored respiration and reduced activity, it was not considered feasible to measure blood pressure via the noninvasive tail-cuff method or to use more complicated techniques to measure pressures in closed-chest mice (in such cases, peak systolic pressures are usually higher). It is noteworthy that our values for LV pressures are similar to those reported in a previous study using similar open-chest techniques.26 Importantly, under the same experimental conditions, there was no

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**TABLE 1. Postmortem Analysis of Aortic-Banded Mice Treated With Rapamycin**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Rapamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham Banded</td>
<td>Sham Banded</td>
</tr>
<tr>
<td>No. of animals</td>
<td>6 8</td>
<td>5 10</td>
</tr>
<tr>
<td>Body weight, g</td>
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<tr>
<td>Tibial length, mm</td>
<td>16.8±0.1</td>
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<tr>
<td>Heart weight, mg</td>
<td>107.5±2.4</td>
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<tr>
<td>Lung weight, mg</td>
<td>157.7±8.1</td>
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</tr>
<tr>
<td>Liver weight, mg</td>
<td>1186±71</td>
<td>1173±107</td>
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<tr>
<td>Heart weight/body wt, mg/g</td>
<td>4.30±0.09</td>
<td>4.43±0.22</td>
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<td>Lung weight/body wt, mg/g</td>
<td>6.32±0.32</td>
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<td>Liver weight/body wt, mg/g</td>
<td>47.2±1.9</td>
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<td>Heart weight/tibial length, mg/mm</td>
<td>6.40±0.15</td>
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<tr>
<td>Lung weight/tibial length, mg/mm</td>
<td>9.39±0.48</td>
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<tr>
<td>Liver weight/tibial length, mg/mm</td>
<td>70.7±4.3</td>
<td>71.1±5.8</td>
</tr>
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</table>

*P<0.05 vs sham-operated mice treated with rapamycin or vehicle. †P<0.05 vs vehicle-treated aortic-banded mice.

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**TABLE 2. Hemodynamic Measurements of Aortic-Banded Mice Treated With Rapamycin**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Rapamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham Banded</td>
<td>Sham Banded</td>
</tr>
<tr>
<td>No. of animals</td>
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<td>5 5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>371±15</td>
<td>399±11</td>
</tr>
<tr>
<td>LV systolic pressure, mm Hg</td>
<td>49.2±4.3</td>
<td>52.2±3.1</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>5.6±0.5</td>
<td>5.6±0.5</td>
</tr>
<tr>
<td>Heart weight/tibial length, mg/mm</td>
<td>6.61±0.14</td>
<td>5.84±0.08</td>
</tr>
</tbody>
</table>

Results are presented as mean±SEM.

*P<0.05 vs sham-operated mice treated with rapamycin or vehicle.
†P<0.05 vs vehicle-treated aortic-banded mice.
Effect of Rapamycin on Myocyte Cell Size

To examine whether rapamycin changes heart size through the regulation of myocyte cell size, the myocyte cross-sectional area of the LV myocardium was measured in hearts from mice subjected to aortic banding for 1 week (Figure 3). The cell area of vehicle-treated aortic-banded mice was increased by 46.7 \(\pm\) 6.0% compared with vehicle-treated sham-operated animals. The cell area of hearts from aortic-banded mice treated with rapamycin was significantly lower than that of vehicle-treated aortic-banded mice. Thus, rapamycin attenuated load-induced increase in myocyte cell size. Both vehicle- and rapamycin-treated sham-operated mice showed a significant difference in LV systolic or diastolic pressures between vehicle-treated aortic-banded mice and rapamycin-treated aortic-banded mice (Table 2). However, the heart weight/tibial length ratio of rapamycin-treated aortic-banded mice was increased by 6.2% compared with rapamycin-treated sham-operated animals. The cell area of hearts from aortic-banded mice was significantly lower than that of vehicle-treated aortic-banded mice. Thus, rapamycin attenuated load-induced increase in myocyte cell size by 57%. Collectively, this suggests that rapamycin attenuated the load-induced increase in heart size primarily through the regulation of myocyte cell size.

Effect of Rapamycin on Cardiac Function of Aortic-Banded Mice

One week after surgery, we examined the effect of rapamycin on cardiac function using echocardiography (Table 3). Rapamycin treatment significantly decreased LV diastolic and systolic diameters in aortic-banded mice. Cardiac contractility, assessed by fractional shortening, was not different between vehicle-treated and rapamycin-treated animals. Thus, rapamycin treatment was associated with a decrease in chamber size and normal systolic function.

Effect of Rapamycin on Fetal Gene Expression in Aortic-Banded Mice

Cardiac hypertrophy in response to pressure overload is associated with reactivation of “fetal” genes.\textsuperscript{27} We previously showed that in cultured rat neonatal myocytes, rapamycin inhibited increases in cell size and protein synthesis stimulated by angiotensin II, but angiotensin II–induced fetal gene expression was not affected by rapamycin.\textsuperscript{12} We examined the expression of ANP and BNP in the hearts from mice 1 week after the operation (Figure 4). There was no significant difference in the amount of ANP or BNP mRNA between the aortic-banded groups with or without rapamycin.

Discussion

mTOR plays a key role in regulating cell growth.\textsuperscript{8} In this study, we examined the effect of rapamycin, a specific inhibitor of mTOR,\textsuperscript{11,28} on pressure overload–induced cardiac hypertrophy. Rapamycin, at a clinically relevant dose, effectively attenuated load-induced cardiac hypertrophy, and this was associated with attenuation of the increase in myocyte cell size. Furthermore, rapamycin did not cause lethality, loss of body weight, or deterioration of cardiac function. To the best of our knowledge, this is the first study to demonstrate that rapamycin can reduce heart size without disturbing cardiac function in vivo.

Rapamycin inhibits angiotensin II and phenylephrine-mediated hypertrophy of cardiac myocytes in vitro, and inhibition of S6K1 has been implicated as a key factor in this response.\textsuperscript{12,13} In the present study, S6K1 was activated significantly in the heart in response to acute pressure.
overload in vivo, and rapamycin completely suppressed load-induced S6K1 activation and S6 phosphorylation. Thus, activation of S6K1 seems to be critical for the development of cardiac hypertrophy in response to pressure overload. Another important effector of mTOR, 4E-BP1, may also modulate this response. Phosphorylation of 4E-BP1 results in release of eIF4E, allowing increased formation of the eIF4F translation factor complexes. Rapamycin can inhibit insulin-induced phosphorylation of 4E-BP1 in cardiomyocytes, and this may affect protein synthesis in the heart.29,30

We also examined the effect of aortic banding and rapamycin on a number of other downstream signaling molecules. JNK1 activity increased with banding and was partially but significantly attenuated by rapamycin treatment. Thus, rapamycin, either directly or indirectly, selectively inhibited one of the MAP kinase pathways. It was reported that rapamycin inhibited JNK1 activation in lymphocytes by an unknown mechanism.31 Phosphorylation of GSK3β, a downstream target of Akt and a negative regulator of heart growth,24,25 was not affected by pressure overload or rapamycin treatment in the present study. By contrast, GSK3β was shown to be inactivated in hearts of rats in response to aortic banding.32 This discrepancy may be explained by the more severe banding conditions used by Haq et al.32

Rapamycin has been used in the treatment of transplant rejection.19 Recently, it was shown that rapamycin-coated stents potently inhibited restenosis after coronary angioplasty.33 Calcineurin inhibitors, such as cyclosporin A and FK506, have been the mainstays of immunosuppression for solid-organ transplantation. However, the nephrotoxicity of calcineurin inhibitors limits their therapeutic benefit.34 Because rapamycin has a unique mechanism of action distinct from that of calcineurin inhibitors and is associated with less nephrotoxicity,35 it (or its derivatives) might be a useful modality to suppress cardiac hypertrophy. In this study, we have shown that pretreatment of rapamycin effectively prevents load-induced overgrowth of the heart. Future studies are necessary to examine whether rapamycin can regress established cardiac hypertrophy and cardiomyopathy. In addition, because the phosphorylation of S6 is believed to be critical for the translation of specific mRNA species, it would be of interest to examine which mRNA species are inhibited by rapamycin.

Acknowledgments
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References

Figure 4. Effect of rapamycin on cardiac “fetal” gene expression. Expression of ANF or BNP was examined by Northern hybridization analysis (left). Loading of RNA was normalized by reprobing membrane with GAPDH. Right, Results of quantitative densitometric analysis. S indicates sham-operated mice; B, aortic-banded mice; n, number of mice. *P<0.05 vs sham-operated animals given vehicle or rapamycin. Mean value of vehicle-treated sham-operated mice was defined as 1 U.

References
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