ANIMAL MODELS

Genetic and Pharmacological Inhibition of Rheb1-mTORC1 Signaling Exerts Cardioprotection against Adverse Cardiac Remodeling in Mice

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Accepted for publication
February 7, 2013.

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A previous study indicated that Rheb1 is required for mammalian target of TOR complex 1 (mTORC1) signaling in the brain. However, the function of Rheb1 in the heart is still elusive. In the present study, we deleted *Rheb1* specifically in cardiomyocytes and found that reduced Rheb1 levels conferred cardioprotection against pathologic remodeling in myocardial infarction (MI) and pressure overload (transverse aortic constriction) mouse models. Cardiomyocyte apoptosis was reduced and mTORC1 activity was suppressed in cardiomyocyte *Rheb1*-deletion mice, suggesting that Rheb1 regulates mTORC1 activation in myocardium. Furthermore, we demonstrated that astragaloside IV (As-IV) could inhibit mTORC1, and As-IV treatment displayed similar protection against MI and transverse aortic constriction as *Rheb1* genetic inhibition. This study indicates that Rheb1 is essential for mTORC1 activation in cardiomyocytes and suggests that targeting Rheb1-mTORC1 signaling, such as by As-IV treatment, may be an effective therapeutic method for treating patients with adverse cardiac remodeling after MI and hypertrophy. (*Am J Pathol* 2013, 182: 2005–2014; <http://dx.doi.org/10.1016/j.ajpath.2013.02.012>)

Congestive heart failure (CHF) is a leading cause of morbidity and mortality worldwide. CHF is commonly caused by pathologic cardiac remodeling after a wide spectrum of cardiovascular diseases, such as hypertension and myocardial infarction (MI).^{1–3} Pathologic cardiac remodeling refers to changes in geometry, mass, volume, and function of the heart after injury to the ventricles. Major hallmarks of this remodeling include enlarged cardiomyocyte size, the buildup of extracellular matrix, and increased cardiomyocyte apoptosis.⁴ Although myocardial remodeling is compensatory initially, it can become maladaptive and eventually lead to CHF if left untreated. Therefore, the relief of adverse myocardial remodeling is a critical therapeutic strategy to treat CHF. Despite the widespread use of β -blockers and inhibitors of the renin-angiotensin-aldosterone system clinically in the past few decades, the incidence of CHF still remains high, highlighting the need for additional understanding of remodeling events.

In recent years, protein synthesis involved in the remodeling process has been given remarkable attention. One important molecule related to protein synthesis is the mechanistic target of rapamycin (TOR) complex 1 or mammalian target of TOR complex 1 (mTORC1) that is involved in cell growth, proliferation, and differentiation. mTORC1 activity is strongly stimulated by upstream Rheb, whereas tuberous sclerosis complex 1/2 negatively regulates mTORC1 by inhibition of Rheb activity.⁵

There are two Rheb isoforms, termed Rheb1 and Rheb2, in mice.⁶ Deletion of *Rheb1* in mice results in embryonic

Supported in part by National Key Basic Research Program of China grants 2011CB943904 and 2012CB966602; National Natural Science Foundation of China grants 31071282, 91019002, 31130037, 81170201, and 81273943 (Z.Y., X.L., and X.C.); and the Open Research Fund of State Key Laboratory of Bioelectronics, Southeast University.

Figure 1 Generation of cardiomyocyte-specific *Rheb1*-deletion mice. **A:** Genotyping of mice by PCR. **B:** Western blot analysis of Rheb in the heart. WT, wild type.

lethality between embryonic day (E) 10.5 and E11.5, whereas loss of *Rheb2* in mice does not affect embryonic development, and mice could survive to adulthood without apparent defects.⁶ In addition, Rheb1 has been shown to be essential for mTORC1 signaling.⁶

To understand the function of Rheb in the heart, we deleted *Rheb1* in cardiomyocytes through Cre-mediated excision in mice. Half loss of Rheb1 conferred cardiac protection against pathologic heart remodeling resulting from MI and pressure overload. Cardiac mTORC1 signaling was disrupted in *Rheb1*-deficient mice. These results demonstrated that Rheb mediates mTORC1 signaling in the heart and that Rheb-mTORC1 signaling is involved in pathologic heart remodeling. Meanwhile, this study suggested that inhibition of Rheb-mTORC1 signaling might be an effective strategy for treating pathologic heart remodeling.

Rapamycin is a potent mTOR inhibitor.⁷ However, recent studies have revealed the adverse effects of rapamycin, such as body weight loss, increased risks of infection and cancer, and diabetes-like symptoms.^{7–11} Therefore, it is clinically significant to screen a new drug targeting mTORC1 signaling with mild adverse effects. In the present study, we found that astragaloside IV (As-IV), extracted from Chinese medicine *Astragalus membranaceus* (Fisch) Bge, could inhibit mTORC1 activity in different cell lines, such as

Figure 2 Loss of *Rheb1* ameliorates cardiac dysfunction and aberrant heart remodeling after MI. **A:** Ratio of heart weight to body weight. **B–E:** Echocardiography measurement. **F:** Masson's staining was used to display fibrotic areas (in blue). **G:** Quantitation of fibrotic area ($n = 3$). **H:** Wheat germ agglutinin staining was used to display the structure and size of cardiomyocytes. Sections were from papillary muscle. **I:** Quantitation of cross areas of cardiomyocytes ($n = 3$). **J:** TUNEL assay. Apoptotic cells are in red. Sections were taken from the border zone of the infarct area. **K:** Quantitation of apoptotic cells ($n = 3$). **L:** H&E staining showed necrosis and inflammation in the border zone of the infarct area 3 days after MI surgery. **M:** Quantitation of necrosis area ($n = 3$). EF, ejection fraction; FS, fractional shortening; LVIDD, left ventricular internal diastolic diameter; SHAM, mice after sham operation. Data are given as means $\pm$ SEM. * $P < 0.01$. Scale bars: 50 $\mu\text{mol/L}$ (H); 30 $\mu\text{mol/L}$ (L).

Figure 3 Deletion of *Rheb1* represses cardiac hypertrophy induced by pressure overload. **A:** Ratio of heart weight to body weight. **B:** H&E staining was used to show the cross section of heart size, and Masson's staining was used to display fibrotic areas (in blue). **C:** Quantitation of collagen distribution ($n = 3$). **D:** Wheat germ agglutinin staining of structure and size of cardiomyocytes. Sections were from papillary muscle. **E:** Quantitation of cross areas of cardiomyocytes ($n = 3$). SHAM, mice after sham operation. Data are given as means $\pm$ SEM. * $P < 0.01$, ** $P < 0.05$. Scale bars: 1 mmol/L (**B**, upper panels); 40 μ m (**B**, lower panels); 50 μ m (**D**).

HeLa and HEK293. Moreover, As-IV was shown to effectively alleviate pathologic heart remodeling and to reduce mTORC1 signaling in mouse models.

Materials and Methods

Mice

Mice with a C57BL/6 genetic background were housed in groups with 12-hour dark/light cycles and with free access to food in accordance with the regulations on mouse welfare and ethics of Nanjing University (Nanjing, China). All the procedures were conducted with relevant authority approval. Ras homolog enriched in brain (*Rheb1*)—floxed mice were as described previously and were maintained on a C57BL/6 genetic background.⁶ To delete *Rheb1* in cardiomyocytes, *Rheb1*-floxed mice were crossed with α -myosin heavy chain (α MHC)—Cre mice, and the progenies were genotyped by PCR. DNA primers for *Rheb1* genotyping were as follows: 5'-GCCGAGAACATCTGTTCCAT-3' and 5'-GGTACC-CACAACCTGACACC-3' to amplify wild-type and 5' floxed alleles, respectively. PCR products for wild-type and floxed alleles are 650 and 850 bp, respectively (Figure 1A).

Cell Culture and As-IV Treatment

As-IV (98% purity) was purchased from Nanjing Zelang Medical Technology Co. Ltd. (Nanjing, China).

293T cells and HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10%

fetal calf serum. These cells were treated with As-IV (1 μ mol/L) or dimethyl sulfoxide and were collected at 36 and 48 hours for Western blot analysis. In parallel, 100 nmol/L rapamycin was applied to these cells for 1 hour for mTOR inhibition.

Ventricular myocytes were prepared from mice (2 to 3 days after birth) with a C57BL/6 genetic background and cultured on Permanox chamber slides (Thermo Scientific Nunc, Waltham, MA) coated with 1% gelatin and 0.0015% laminin solution, as previously described.¹² Afterward, these cells were treated with dimethyl sulfoxide, isoproterenol (ISO) (10 μ mol/L) + dimethyl sulfoxide, or ISO (10 μ mol/L) + As-IV (1 μ mol/L) for 2 days. After treatment, these cells were collected for immunofluorescence staining and Western blot analysis.

Mouse Models of MI and TAC and Administration of As-IV

MI and transverse aortic constriction (TAC) were generated following a method reported previously in mice.^{13,14} Briefly, male mice aged 8 to 12 weeks weighing 25 to 30 g were anesthetized i.p. with 0.4 to 0.75 mg/g of body weight tribromoethanol [Avertin (2,2,2-tribromoethanol; Sigma-Aldrich, St. Louis, MO)]. An endotracheal tube was introduced into the trachea, and a volume-cycled rodent respirator (model 683; Harvard Apparatus, Holliston, MA) provided positive pressure ventilation at 2 to 3 mL per cycle with a respiratory rate of 120 cycles per minute. For MI surgery, the thoracic cavity was opened and ligation of the left anterior descending coronary artery was performed

Figure 4 Cardiac ablation of *Rheb1* impairs mTORC1 signaling. **A:** Heart tissues were dissected from a remote area for Western blot analysis of S6 and 4E-BP1 phosphorylation (P) in mice with MI. **B:** Quantitative study ($n = 3$). **C:** Western blot analysis of S6 and 4E-BP1 phosphorylation in heart tissues of mice after TAC. **D:** Quantitative study ($n = 3$). **E:** Western blot analysis of S6K, Akt, p38, and ERK phosphorylation in heart tissues (remote area) of MI mice. **F:** Quantitative study ($n = 3$). **G:** Western blot analysis of S6K, Akt, p38, and ERK phosphorylation in heart tissues of TAC mice. **H:** Quantitative study ($n = 3$). SHAM, mice after sham operation. Data are given as means $\pm$ SEM. * $P < 0.01$, ** $P < 0.05$.

using a 7-0 silk suture 3 to 4 mm from the tip of the left auricle. The chest was closed using a continuous 6-0 prolene suture, followed by a 4-0 polyester suture to close the skin. TAC surgery was performed by tying a 7-0 polypropylene suture around the transverse aorta against a 27-gauge needle to yield a narrowing 0.4 mm in diameter.

As-IV was administrated to mice i.p. at a dose of 2 mg/kg of body weight for 21 days after MI and TAC surgery.

Echocardiography

Echocardiography was performed using a Vevo 770 UBM system (VisualSonics Inc., Toronto, ON, Canada) that possesses a single-element mechanical transducer with a center frequency of 30 MHz and a frame rate of 30 Hz.⁷ The spatial resolution of B-mode imaging was approximately 115 μ m (lateral) by approximately 55 μ m (axial). Mice were anesthetized with Avertin as described for MI surgery. The body temperature of mice was monitored using a rectal thermometer and was maintained between 36°C and 38°C. The heart rate was maintained between 350 and 450 beats/min. After measurement, cardiac output values, such as ejection fraction, fractional shortening, and left ventricular internal diastolic diameter, were calculated according to the guidelines accompanying the Vevo 660 UBM system.⁷

Western Blot Analysis

Heart tissues were collected from the MI remote area in the left ventricle. Tissue lysates were prepared in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 10% glycerol, 20 mmol/L glycerophosphate, 1% NP40, 5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L Na₃VO₄, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 1 mmol/L dithiothreitol, 50 mmol/L NaF, and 4 μ mol/L leupeptin, pH 8.0). Samples were resolved by 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with Tris-buffered saline and Tween 20 (50 mmol/L Tris, 150 mmol/L NaCl, and 0.5 mmol/L Tween 20, pH 7.5) and then were incubated with primary antibodies overnight. The following antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA): Rheb (#4935), S6 (#2317), phosphor-S6 (S235/236) (#2211), phosphor-S6 (S240/244) (#2215), 4E-BP1 (#9452), phosphor-4E-BP1 (T37/46) (#9459), phosphor-4E-BP1(S65) (#9451), p70S6K (#9202), phospho-p70S6K (Thr389) (#9206), total Akt (#9272), phospho-Akt (Thr308) (#9275), phospho-Akt (Ser473) (#9271), extracellular signal-regulated kinase (ERK; #9102), phospho-ERK (Tyr202/Tyr204) (#9101), p38 (#9212), and phospho-p38 (Thr180/Tyr182) (#9211). Pan-

Figure 5 Identification of As-IV as a natural inhibitor of mTORC1 signaling. **A:** The structure of As-IV. **B:** 293T cells were incubated with rapamycin (RAPA) or As-IV and were collected for Western blot analysis. **C:** Quantitative study ($n = 3$). **D:** HeLa cells incubated with RAPA or As-IV were collected for Western blot analysis. **E:** Quantitative study ($n = 3$). **F:** Cardiac troponin T (cTnT) staining (green) of cardiomyocytes. **G:** Quantitative study ($n = 3$). **H:** Cardiomyocytes from young mice 2 to 3 days after birth incubated with As-IV and ISO were collected for Western blot analysis. **I:** Quantitative study ($n = 3$). Data are given as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

actin antibody (#MS-1295-P0) and horseradish peroxidase–linked secondary antibodies (#31460 and #31430) were purchased from Thermo Scientific (Rockford, IL).

Histologic Analysis and Immunofluorescence Staining

H&E and Masson's staining and immunofluorescence were performed as described previously.^{7,15,16} Briefly, heart samples were first washed with cold PBS and then were fixed in 4% paraformaldehyde at 4°C. The samples were processed successively by i) a 30-minute wash in PBS at 4°C; ii) 15-minute incubations in 30%, 50%, 75%, and 85% ethanol and then two 10-minute incubations in 95% and 100% ethanol at room temperature; iii) three 10-minute incubations in xylene at room temperature; iv) a 20-minute incubation in paraffin/xylene (dilution 1:1) at 65°C; and v) three 30-minute incubations in fresh paraffin at 65°C. The processed samples were then embedded in paraffin and sectioned (6 μ m thick), and the sections were stained. Immunofluorescence staining was performed using anti–cardiac troponin T antibody and anti–wheat germ agglutinin

antibody at 4°C overnight. Fluorescence microscopy images were obtained using a research fluorescence microscope (Olympus America Inc., Center Valley, PA) equipped with a digital camera. Images were collected and recorded using Photoshop version 5.0 (Adobe Systems Inc., San Jose, CA) on an IBM R52 computer (IBM, Armonk, NY).

TUNEL Assay

TUNEL assay was performed as previously reported.^{7,15} Briefly, the sections were treated with 20 μ g/mL of proteinase K and were incubated with terminal deoxynucleotidyl transferase and biotinylated deoxyuridine triphosphate.

Statistical Analysis

Data are presented as means $\pm$ SEM values. For comparisons between two groups, statistical significance was determined using the unpaired two-tailed Student's *t*-test. A $P < 0.05$ was considered statistically significant, and a $P < 0.01$ was considered statistically very significant.

Figure 6 As-IV improves cardiac function and protected against MI. **A**: Ratio of heart weight to body weight. **B–E**: Echocardiography measurement. **F**: Masson's staining was used to display fibrotic areas (in blue). **G**: Quantitative study ($n = 3$). **H**: Wheat germ agglutinin staining was used to display the structure and size of cardiomyocytes. Sections were from papillary muscle. **I**: Quantitation of cross areas of cardiomyocytes ($n = 3$). **J**: TUNEL assay. Apoptotic cells are shown in red. Sections were taken from the border zone of the infarct area. **K**: Quantitative study ($n = 3$). **L**: H&E staining was used to show necrosis and inflammation in the border zone of the infarct area 3 days after MI surgery. **M**: Quantitation of the necrosis area ($n = 3$). EF, ejection fraction; FS, fractional shortening; LVIDD, left ventricular internal diastolic diameter; SHAM, mice after sham operation. Data are given as means $\pm$ SEM. * $P < 0.01$. Scale bars: 1 μ m (**F**); 50 μ m (**H**); 30 μ m (**L**).

Results

Generation of Cardiomyocyte-Specific *Rheb1*-Deletion Mice

Rheb1-floxed mice were as previously reported and were maintained in a C57/B6 genetic background.⁶ To delete *Rheb1* specifically in cardiomyocytes, female *Rheb1*-floxed mice (*Rheb1*^{F/F}) were crossed with male α MHC-Cre mice to obtain *Rheb1*^{F/+}; α MHC-Cre mice. Mice were genotyped by PCR (Figure 1A), and loss of *Rheb1* in heart tissue was confirmed by Western blot analysis. The results revealed that *Rheb1* protein levels were significantly reduced in *Rheb1*-deletion mice compared with controls (Figure 1B).

Loss of *Rheb1* Ameliorates Cardiac Dysfunction and Aberrant Heart Remodeling after MI

Disruption of both alleles of *Rheb1* in cardiomyocytes (*Rheb1*^{F/F}; α MHC-Cre) resulted in mouse mortality at

a young age (data not shown). However, half deletion of *Rheb1* in cardiomyocytes (*Rheb1*^{F/+}; α MHC-Cre) had little effect on heart function and mouse survival. To investigate the role of *Rheb1* in cardiac protection, we first performed MI surgery in control and *Rheb1*-deletion mice to induce myocardial damage. Three weeks after MI, the heart weight to body weight ratio was increased significantly in control mice (Figure 2A). However, loss of *Rheb1* profoundly reduced the heart weight to body weight ratio (Figure 2A). Echocardiography measurement indicated that heart systolic function was markedly impaired in control MI mice and well preserved in *Rheb1*-deletion mice (Figure 2, B and C). In addition, loss of *Rheb1* greatly reduced the left ventricular internal diastolic diameter compared with control MI mice (Figure 2, D and E). Histologic analysis displayed substantially reduced fibrosis area, cardiomyocyte size, and cell apoptosis in *Rheb1*-deletion mice compared with control MI mice (Figure 2, F–K). Finally, we investigated necrosis 3 days after MI and found that necrosis and inflammation in the border area was reduced in *Rheb1*-

Figure 7 As-IV suppresses cardiac remodeling induced by TAC. **A:** Ratio of heart weight to body weight. **B:** H&E staining was used to show the cross section of heart size, and Masson's staining was used to display fibrotic area (in blue). **C:** Quantitative study ($n = 3$). **D:** Wheat germ agglutinin staining was used to display the structure and size of cardiomyocytes. Sections were from papillary muscle. **E:** Quantitation of cross areas of cardiomyocytes ($n = 3$). SHAM, mice after sham operation. Data are given as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$. Scale bars: 1 mmol/L (**B**, upper panels); 40 μ m (**B**, lower panels); 50 μ m (**D**).

deletion mice compared with control MI mice (Figure 2, L and M).

Taken together, these results indicated that genetic ablation of *Rheb1* dramatically suppressed pathologic heart remodeling and improved heart function after MI. Thus, *Rheb1* functions to protect against myocardial damage in the heart.

Deletion of *Rheb1* Represses Cardiac Hypertrophy Induced by Pressure Overload

Next, we investigated the function of *Rheb1* in heart hypertrophy. Control and *Rheb1*-deletion mice were challenged with TAC to induce heart hypertrophy. Three weeks after TAC surgery, cardiac hypertrophy was apparent in control mice but was significantly repressed in *Rheb1*-deletion mice (Figure 3, A and B). Masson's staining revealed extensive cardiac fibrosis in control mice, whereas it was hard to detect cardiac fibrosis in *Rheb1*-deletion mice (Figure 3, B and C). In control mice, TAC substantially increased cardiomyocyte size revealed by immunofluorescence staining, but this effect was less severe in *Rheb1*-deletion mice (Figure 3, D and E).

Collectively, these results indicated that *Rheb1* was involved in pathologic remodeling and hypertrophic cardiomyopathy.

Cardiac Ablation of *Rheb1* Impairs mTORC1 Signaling

We examined mTORC1 signaling in the heart tissue after MI and TAC surgery. Phosphorylation levels of 4E-BP1 and

S6, indicators of mTORC1 signaling, were profoundly enhanced in control mice after MI and TAC (Figure 4, A–D). In contrast, deletion of *Rheb1* markedly reduced the phosphorylation levels of 4E-BP1 and S6, indicating impaired mTORC1 signaling (Figure 4, A–D). Furthermore, we examined the signaling pathways of S6K, Akt, ERK, and p38. The results showed that phospho-S6K levels were significantly reduced in *Rheb*-deletion mice compared with control MI and TAC mice, whereas phosphorylation levels of Akt, ERK, and p38 were not changed (Figure 4, E–H).

Identification of As-IV as a Natural Inhibitor of mTORC1 Signaling

Rapamycin and its derivatives have been extensively studied, and they are well accepted as potent mTOR inhibitors.⁷ Nonetheless, recent studies indicated that these chemicals might cause severe adverse effects, such as glucose metabolic disorder, gastrointestinal inflammation, and immunosuppression.^{7–11} Therefore, it is necessary to search for better natural products to substitute for rapamycin and its derivatives for clinical application. We tested As-IV, a monomeric compound isolated from Chinese herbs that have been demonstrated to be effective in treating human cardiovascular diseases, and found that it could inhibit mTORC1 signaling; the structure of As-IV is shown in Figure 5A. Treatment of HEK293T and HeLa cells with As-IV significantly reduced 4E-BP1 and S6 phosphorylation (Figure 5, B–E). ISO treatment was shown to induce cardiomyocyte hypertrophy.^{17,18} We isolated cardiomyocytes

Figure 8 As-IV inhibits mTORC1 signaling in the heart. **A:** Heart tissues were dissected from a remote area for Western blot analysis of S6 and 4E-BP1 phosphorylation (P) in mice with MI. **B:** Quantitative study ($n = 3$). **C:** Western blot analysis of S6 and 4E-BP1 phosphorylation in heart tissues of mice after TAC. **D:** Quantitative study ($n = 3$). **E:** Western blot analysis of S6K, Akt, p38, and ERK phosphorylation in heart tissues (remote area) of MI mice. **F:** Quantitative study ($n = 3$). **G:** Western blot analysis of S6K, Akt, p38, and ERK phosphorylation in heart tissues of TAC mice. **H:** Quantitative study ($n = 3$). WT, wild type. Data are given as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

from young mice 2 to 3 days after birth and cultured these cells. ISO treatment of these cells for 2 days caused hypertrophy (Figure 5, F and G). Co-treatment with ISO and As-IV significantly reduced cardiomyocyte hypertrophy (Figure 5, F and G). Western blot analysis showed that ISO treatment activated mTORC1 signaling, which was blocked by As-IV (Figure 5, H and I).

As-IV Improves Cardiac Function and Protects against MI

Next, we tested the effects of As-IV in the MI mouse model. We first investigated whether As-IV had toxic effects in mice. Mice were treated with As-IV for 6 weeks. Afterward, body weight and heart function were measured, and the results indicated that As-IV did not impair heart function and reduce body weight (Supplemental Figure S1). After MI surgery, mice were administered As-IV for 21 days; the heart weight to body weight ratio was significantly reduced in As-IV-treated mice compared with vehicle-treated mice (Figure 6A). As-IV treatment also dramatically improved heart function as indicated by ejection fraction percentage and fractional shortening percentage (Figure 6, B and C). Meanwhile, heart dilatation was greatly suppressed after

As-IV administration (Figure 6, D and E). In addition, fibrotic area, cardiomyocyte hypertrophy, and cell apoptosis were markedly reduced in As-IV-treated mice compared with vehicle-treated mice (Figure 6, F–K). We also investigated necrosis 3 days after MI and found that necrosis and inflammation in the border area was reduced in As-IV-treated mice compared with control MI mice (Figure 6, L and M). These results demonstrated that As-IV effectively improved heart function and protected against adverse cardiac remodeling after MI.

As-IV Suppresses Cardiac Remodeling Induced by TAC

We further investigated whether As-IV could inhibit cardiac remodeling induced by TAC. Mice underwent TAC surgery and were subsequently administered As-IV for 21 days. Figure 7A shows that the heart weight to body weight ratio was smaller in As-IV-treated mice than in vehicle-treated mice. Histologic analysis revealed less hypertrophic myocardium and reduced cardiomyocyte size in As-IV-treated mice compared with controls (Figure 7, B–E). Taken together, these results indicated that As-IV effectively suppressed cardiac remodeling induced by TAC.

As-IV Inhibits mTORC1 Signaling in the Heart

Heart tissues were collected from As-IV- and vehicle-treated mice 21 days after MI and TAC for Western blot analysis. mTORC1 signaling was analyzed, and the results showed that As-IV treatment substantially blocked mTORC1 signaling, as indicated by reduced 4E-BP1 and S6 phosphorylation levels (Figure 8, A–D). Furthermore, we examined the signaling pathways of S6K, Akt, ERK, and p38. The results showed that phospho-S6K levels were significantly reduced in As-IV-treated mice compared with control MI and TAC mice, whereas phosphorylation levels of Akt, ERK, and p38 were not changed (Figure 8, E–H).

Discussion

In the present study, we provided the first genetic evidence that ablation of *Rheb1* improved heart function and suppressed pathologic heart remodeling after MI and TAC. mTORC1 signaling activation was repressed by loss of *Rheb1* in the heart after MI and TAC, suggesting that Rheb-mediated mTORC1 signaling is involved in aberrant heart remodeling. Subsequently, we reported the identification of As-IV, a natural product, as a potent inhibitor against mTORC1 signaling and that As-IV rendered a beneficial effect on cardiac protection against MI and pressure overload. This study not only elucidated the function of Rheb1 in cardiac mTORC1 signaling and remodeling but also provided supporting evidence for the clinical applications of As-IV to treat cardiomyopathy.

Rapamycin and its derivatives are potent inhibitors against mTOR signaling. However, severe adverse effects of rapamycin and its derivatives limit their clinical application and prompt the search for better substitutes.^{7–11} *Astragalus membranaceus* Bge, a Chinese herb, has been widely prescribed to improve heart function.^{19–21} However, the underlying mechanisms in treating cardiovascular disease are elusive. In this study, we identified As-IV, a monomeric compound isolated from *Astragalus membranaceus* Bge, as a novel inhibitor against mTORC1 signaling. The results of this study suggest that *Astragalus membranaceus* Bge executes cardiac protection, in part through inhibition of mTORC1 signaling.

Previously, it was reported that *S6k1/S6k2* DKO mice showed no heart phenotype.²² However, overexpression of *S6k* in cardiomyocytes substantially increased heart size, leading to hypertrophy in mice. Thus, it seems that loss of function of S6K has little effect on heart morphology but that gain of function does. Recently, Condorelli and colleagues²³ deleted *mTor* in cardiomyocytes and found dilated cardiomyopathy. This study demonstrated that loss of *mTor* impaired heart morphology and function. They also found that the protein levels of 4E-BP1 were markedly elevated, suggesting that 4E-BP1 is the major downstream target of mTOR in regulating heart morphology and function. However, in another study by Shen et al,²⁴ it was found

that mTOR kinase-dead transgenic mice did not manifest morphologic changes. In mTOR kinase-dead transgenic mice, the protein levels of 4E-BP1 were not significantly changed, and this may be the reason why these mice did not show the heart morphologic phenotype as *mTor*-deletion mice displayed. Furthermore, we found that half loss of *Rheb1* (*Rheb1*^{+/-}) had little effect on Akt S473 phosphorylation, whereas complete loss of *Rheb1* (*Rheb1*^{-/-}) significantly enhanced the phosphorylation levels of Akt S473, probably through an mTORC1-Akt-negative feedback mechanism.⁷

In summary, we demonstrated that activation of mTORC1 is mediated by Rheb in the heart, which is involved in cardiac remodeling. Genetic and pharmacologic inhibition of Rheb-mTORC1 renders beneficial effects against cardiomyopathy caused by MI and pressure overload. As-IV, a natural product, can inhibit mTORC1 signaling and may be developed as a potential drug for treating pathologic heart remodeling and other diseases related to mTORC1 hyperactivation.

Acknowledgment

We are grateful to Dr. Bo Xiao (Sichuan University of China) for providing the *Rheb1*-floxed mice.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.02.012>.

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