S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt

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In the present study, we observed a rapid and robust activation of the ribosomal protein S6K (S6 kinase) provoked by MI (myocardial infarction) in mice. As activation of S6K promotes cell growth, we hypothesized that increased S6K activity contributes to pathological cardiac remodelling after MI and that suppression of S6K activation may prevent aberrant cardiac remodelling and improve cardiac function. In mice, administration of rapamycin effectively suppressed S6K activation in the heart and significantly improved cardiac function after MI. The heart weight/body weight ratio and fibrotic area were substantially reduced in rapamycin-treated mice. In rapamycin-treated mice, decreased cardiomyocyte remodelling and cell apoptosis were observed compared with vehicle-treated controls. Consistently, inhibition of S6K with PF-4708671 displayed similar protection against MI as rapamycin. Mechanistically, we observed significantly enhanced Thr308 phosphorylation and activation of Akt in rapamycin- and PF-4708671-treated hearts. Cardiomyocyte-specific deletion of PDK1 (phosphoinositide-dependent kinase 1) and Akt1/3 abolished cardioprotection after MI in the presence of rapamycin administration. These results demonstrate that S6K inhibition rendered beneficial effects on left ventricular function and alleviated adverse remodelling following MI in mice by enhancing Akt signalling, suggesting the therapeutic value of both rapamycin and PF-4708671 in treating patients following an MI.

Key words: Akt, myocardial infarction, PDK1 (phosphoinositide-dependent kinase 1), PF-4708671, rapamycin, S6 kinase (S6K).

INTRODUCTION

MI (myocardial infarction) is one of the most common heart diseases that cause morbidity and mortality worldwide. Following MI, LV (left ventricular) remodelling occurs and progresses to ventricular dilation, eventually leading to heart failure [1–3]. Therefore understanding the mechanisms underlying post-MI heart remodelling is important for effective retardation against pathological LV remodelling and improvement of heart function [1–3].

The mTOR (mammalian target of rapamycin) signalling network regulates cell proliferation, growth and survival, and is involved in metabolic regulation and tumour transformation [4–7]. Because increased mTOR signalling is detected in a variety of human diseases, including carcinoma, small molecules that target mTOR have been developed and are currently being tested in Phase II/III of clinical trials [8]. These small molecules are mainly rapamycin and its analogues, as well as its derivatives, such as RAPA, CCI-779, RAD001 and AP23573 [9,10]. All of these compounds inhibit mTOR kinase activity and, as a result, activation of its downstream substrate, S6K (S6 kinase), is blocked, which results in diminished protein synthesis and reduced cell growth. Patients with solid or haematological tumours are being treated with these compounds in several clinical trials [9,10].

Rapamycin’s properties against cell growth have been utilized for cardiovascular benefit, as stents impregnated with rapamycin effectively reduce coronary restenosis [11,12]. There have been studies showing that rapamycin significantly attenuates both cardiomyocyte and heart hypertrophy induced by growth factors, hormones or overload and improves cardiac function [13–16]. These studies have indicated that S6K inhibition repressed cardiomyocyte growth. Nonetheless, the role of S6K inhibition in cardiac protection against MI is still elusive.

In the present study, we first report that S6K was rapidly and robustly activated after MI. We hypothesized that increased S6K activity contributed to pathological cardiac remodelling after MI, and that suppression of S6K activation might prevent aberrant cardiac remodelling and improve cardiac function. Administration of rapamycin and PF-4708671, a recently characterized S6K inhibitor [17], effectively inactivated S6K and reduced LV remodelling, and significantly improved heart function after MI. The results of the present study suggest the potential therapeutic value of S6K inhibitors in the treatment of patients following an MI.

MATERIALS AND METHODS

Mice

Mice on a C57BL/6 genetic background were housed in groups with 12 h dark/light cycles and with free access to food in accordance with the regulations on mouse welfare and ethics of

Abbreviations used: αMHC, α-myosin heavy chain; EF, ejection fraction; ERK, extracellular-signal-regulated kinase; FS, fractional shortening; GSK, glycogen synthase kinase; IF, immunofluorescence; LV, left ventricular; LVDD, LV internal diameter at end-diastole; MI, myocardial infarction; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; PDK1, phosphoinositide-dependent kinase 1; Pi3K, phosphoinositide 3-kinase; PRAS40, proline-rich Akt substrate of 40 kDa; S6K, S6 kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; WGA, wheat germ agglutinin.

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Nanjing University. All procedures were conducted with relevant authority approval. PDK1 (phosphoinositide-dependent kinase 1)-floxed mice were as described previously and were maintained on a C57BL/6 genetic background [18]. To delete PDK1 in cardiomyocytes, PDK1-floxed mice were crossed with αMHC (α-myosin heavy chain)-Cre mice and the progenies were genotyped by PCR. Akt3-knockout mice were as described previously [19–21]. Generation of Akt1-floxed mice will be described elsewhere.

Mouse model of MI

MI was generated following a method reported previously in mice [22]. Briefly, male mice, 8–12 weeks of age and weighing from 25 to 30 g, underwent left coronary artery ligation. Mice were anaesthetized intraperitoneally with Avertin (0.4–0.75 mg/g of body weight). An endotracheal tube was introduced into the trachea and a volume-cycled rodent respirator (model 683; Harvard Co.) provided positive pressure ventilation at 2–3 ml/cycle and a respiratory rate of 120 cycles/min. After the thoracic cavity was opened, ligation of the left anterior descending coronary artery was performed with a 7-0 silk suture 3–4 mm from the tip of the left auricle. The chest was closed with a continuous 6-0 prolene suture, followed by a 4-0 polyester suture to close the skin.

Echocardiography

Echocardiography was performed using a Vevo 660 UBM system (VisualSonics) that possesses a single-element mechanical transducer with a centre frequency of 30 MHz and a frame rate of 30 Hz. The spatial resolution of B-mode imaging was ∼115 μm (lateral) by ∼55 μm (axial). Mice were anaesthetized with Avertin as described for MI surgery. The body temperature of mice was monitored using a rectal thermometer and was maintained between 36 and 38 °C. The heart rate was maintained between 350 and 450 beats/min. After measurement, the cardiac output values such as EF (ejection fraction), FS (fractional shortening) and LVIDD (LV internal diameter at end-diastole) were calculated according to the guidelines accompanying the Vevo 660 UBM system.

Administration of rapamycin and PF-4708671

Rapamycin was dissolved in methanol and subsequently diluted in Ringer’s solution. The solution was administered intraperitoneally daily. The vehicle (Ringer’s solution) was administered as a control. The doses of rapamycin were chosen based on previous findings in the literature [23]. The lowest dose of 2 mg/kg of body weight per day in mice is equivalent to a dose of 0.17 mg/kg of body weight per day in humans when normalized by body surface area. PF-4708671 (Tocris Bioscience) was dissolved in ethanol and subsequently diluted in soya bean oil [17]. We found that a dose of 75 mg/kg of body weight per day (intraperitoneal administration) was sufficient to inhibit S6K activation in mouse hearts and this dose was used in the present study.

Mice were treated with rapamycin or the vehicle for 1 week and MI induction was performed subsequently. These mice were randomly divided into four groups: (i) sham vehicle (group I); (ii) sham rapamycin (10 mg/kg of body weight per day, group II); (iii) MI vehicle (group III); and (iv) MI rapamycin (groups IV–VIII). The MI rapamycin groups were divided further into groups of 10 mg/kg of body weight per day (group IV), 5 mg/kg of body weight per day (groups V and VIII) and 2 mg/kg of body weight per day (group VI and VII). At 1 day after MI, PF-4708671 was administered daily to mice for 6 days. Echocardiography was performed on these mice at different time points and the mice were killed. Heart weight and body weight were recorded. Heart samples were stored at −80 °C for Western blot analysis.

Western blot analysis

Heart tissues (whole heart or heart tissues from the infarct, border and remote areas) were dissected and snap-frozen in liquid nitrogen until use. Tissue lysates were prepared in lysis buffer [20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 20 mM 2-mercaptoethanol, 1% Nonidet P40, 5 mM EDTA, 0.5 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM PMSF, 1 mM benzamidine, 1 mM DTT (dithiothreitol), 50 mM sodium fluoride and 4 mM leupeptin]. Proteins were resolved by SDS/PAGE (10% gels) and transferred on to PVDF membranes (Millipore). Membranes were blocked with TBST [Tris-buffered saline containing Tween; 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.5 mM Tween 20] and then incubated overnight with primary antibodies. The following antibodies were purchased from Cell Signaling Technology: phospho-70S6K (cat. no. 9202), phospho-p70S6K (Thr389) (cat. no. 9205), S6 (cat. no. 2317), phospho-S6 (Ser235/Ser240) (cat. no. 2211), total Akt (cat. no. 9272), phospho-Akt (Thr406) (cat. no. 9275), phospho-Akt (Ser473) (cat. no. 2927), ERK (extracellular-signal-regulated kinase) (cat. no. 9102), phospho-ERK ( Tyr202/Tyr204) (cat. no. 9101), p38 (cat. no. 9212), phospho-p38 (Thr180/Tyr182) (cat. no. 9211), phospho-GSK (glycogen synthase kinase)-3α/β (Ser74/Ser87) (cat. no. 9331), GSK-3α (cat. no. 9339), PRAS40 (proline-rich Akt substrate of 40 kDa) (cat. no. 2610), phospho-PRAS40 (Thr446) (cat. no. 2640) and α-β-tubulin (cat. no. 2148). The following rabbit monoclonal antibodies were purchased from Epitomics: phospho-Akt (Thr406) (cat. no. 2214-1), phospho-p70S6K (Thr409) (cat. no. 1175-1) and PDK1 (cat. no. 1624-1). The LC-3 antibody (cat. no. AP-1802a) was purchased from Abgent. The pan-actin antibody (cat. no. MS-1295-P0) and HRP (horseradish peroxidase)-linked secondary antibodies (cat. no. 31460 and cat. no. 31430) were purchased from Thermo Scientific.

Histology and immunofluorescence staining

The protocols for H&E (haematoxylin/eosin) staining, Masson’s staining and IF (immunofluorescence) were as described previously [21,22,24]. Briefly, heart samples were first washed with ice-cold PBS and then fixed in 4% PFA (paraformaldehyde) at 4°C. The samples were processed successively by: (i) a 30 min wash in PBS at 4°C; (ii) 15 min each in 30%, 50%, 75% and 85% ethanol, and then 2× 10 min in 95% and 100% ethanol at room temperature (25°C); (iii) 3× 10 min in xylene at room temperature; (iv) 20 min in paraffin/xylene (1:1) at 65°C; and (v) 3× 30 min in fresh paraffin at 65°C. The processed samples were then embedded in paraffin, sectioned (6-μm-thick sections) and the sections were stained. IF staining was performed using an anti-WGA (wheat germ agglutinin) antibody at 4°C overnight. Fluorescence microscopy images were obtained with a Research Fluorescence Microscope (Olympus) equipped with a digital camera. Images were collected and recorded using Adobe Photoshop® 5.0 on an IBM R52 computer.

TUNEL [TdT (terminal deoxynucleotidyltransferase)-mediated dUTP nick-end labelling] assay

The TUNEL assay was performed as described previously [21]. Briefly, sections were treated with protease K (20 μg/ml) and incubated with TdT and biotinylated dUTP.
similar to those of sham controls at 7 days after MI (Figure 1).

Activated 2 days after MI and its activation decreased to levels comparison with the sham controls, S6K was found to be robustly statistically significant and were carried out. A value of one-way ANOVA followed by Tukey’s multiple-comparison test $P$-S6KT389 represents S6K activation. ($A$) Detection of S6K activation. $P$-S6KT144 represents S6K activation. ($B$) Detection of Akt activation. $P$-Akt$^{S473}$ and $P$-Akt$^{T308}$ represent Akt activation. $d$, day; Non, non-operated.

Statistical analysis

Results are means ± S.E.M. Statistical analyses were performed using GraphPad PRISM 5.0. For comparisons between two groups, statistical significance was determined using an unpaired two-tailed Student’s $t$ test. For comparisons of multiple groups, one-way ANOVA followed by Tukey’s multiple-comparison test were carried out. A value of $P < 0.05$ (*) was considered statistically significant and $P < 0.01$ (**) was statistically very significant.

RESULTS

S6K was rapidly and robustly activated after MI

We examined Akt and S6K activation in hearts at 2 and 7 days after MI [25,26]. Although activation of Akt (indicated by phosphorylation at Ser473 and Thr308) was slightly increased in comparison with the sham controls, S6K was found to be robustly activated 2 days after MI and its activation decreased to levels similar to those of sham controls at 7 days after MI (Figure 1).

Rapamycin effectively inhibited S6K activation and was not detrimental to heart function

Activation of S6K triggers cellular programmes to promote cell growth, which may contribute to pathological cardiac remodelling provoked by MI [27]. Therefore we hypothesized that inhibition of S6K activation by rapamycin may suppress the cell growth programme and prevent abnormal cardiac remodelling, eventually leading to improved heart function after MI.

First, we tested the toxicity of rapamycin in mice. Rapamycin was given to mice at a dose of 10 mg/kg of body weight per day for 10 weeks. Hearts were dissected from these mice and activation of S6K, together with its downstream target S6, was examined [27]. The results indicated that activation of S6K and phosphorylation of S6 were effectively blocked (Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/441/bj4410199add.htm).

Treatment of mice with rapamycin slightly reduced body weight (Supplementary Figure S1C), as reported previously [28]. We found that the heart weight fell in proportion to the body weight, and the heart weight/body weight ratio was equal between vehicle- and rapamycin-treated groups (Supplementary Figure S1D). Moreover, heart function was found to be unaffected by rapamycin treatment, as measurement of the EF failed to detect a difference between the two groups (Supplementary Figure S1E). Collectively, these results suggest that long-term administration of rapamycin in mice was relatively safe and had no adverse effects on heart function.

Rapamycin and the S6K inhibitor PF-4708671 alleviated heart remodelling and improved heart function after MI

Two sets of groups of mice, sham (groups I and II) and MI (groups III–VIII), were treated with vehicle or rapamycin as indicated in Figure 2(A). Mice in groups IV, V and VI were given rapamycin at doses of 10, 5 or 2 mg/kg of body weight per day respectively for 5 weeks (1 week before and 4 weeks after MI) (Figure 2A). Mice in group VII were only treated with rapamycin for 4 weeks after MI to study whether there was a difference between pre- and post-MI rapamycin treatment and post-MI rapamycin treatment (Figure 2A). Mice in group VIII were only treated with rapamycin for 1 week after MI to test for the effects of short-term usage (Figure 2A).

Administration of rapamycin at doses from 2 to 10 mg/kg of body weight per day for 5 weeks had a similar effect on body weight reduction to that shown in Supplementary Figure S1(C) (Figure 2B). However, treatment with rapamycin at a dose of 2 mg/kg of body weight per day for 4 weeks post-MI and short-term usage of rapamycin for 1 week had no apparent effect on body weight loss (Figure 2B).

For the two sham groups (groups I and II), the heart weight/body weight ratio was comparable (Figure 2C), a similar effect as shown in Supplementary Figure S1(D). Among the six MI groups (III–VIII), group III was not treated with rapamycin and the heart weight/body weight ratio in this group was the highest, indicating heart remodelling provoked by MI (Figure 2C). Treatment with rapamycin for 4–5 weeks significantly suppressed heart remodelling after MI, as indicated by the heart weight/body weight ratio (Figure 2C). In addition, short-term administration of rapamycin (group VIII) had no effects in preventing heart hypertrophy (Figure 2C).

At 4 weeks after MI, heart function was considerably impaired, as indicated by the EF measurements (group III in Figure 2D). Treatment with rapamycin greatly improved heart function at 4 weeks after MI (groups IV–VII in Figure 2D). However, short-term usage of rapamycin failed to improve heart function (group VIII in Figure 2D). Similar effects were observed with the FS measurements (Figure 2E).

An increase in LVIDD is an index for cardiac dilation. LVIDD was highest in group III among all of the eight groups, but was significantly smaller in groups IV, V and VI (Figure 2F). Groups VII and VIII had an LVIDD similar to group III (Figure 2F).

S6K is one of the major downstream effectors of mTOR. To study whether rapamycin had a function in post-MI hearts through inhibition of S6K, we tested PF-4708671, a recently characterized S6K inhibitor in mice [17]. PF-4708671 was found to effectively improve heart function and reduce heart dilation (Figures 2G–2I).

Taken together, these results indicate that rapamycin and PF-4708671 alleviated heart remodelling and improved heart function after MI.

Rapamycin and the S6K inhibitor PF-4708671 reduced fibrotic area, cardiomyocyte remodelling and apoptosis

Rapamycin administration substantially reduced the fibrotic area (groups V–VII in Figures 3A and 3B). A smaller LV volume in rapamycin-treated mice than that in vehicle-treated mice was shown by echocardiography (Figures 3Bi and 3Bj). At 4 weeks after MI, the structure of cardiomyocytes was disarrayed and the size of cardiomyocytes increased significantly in the left ventricle of vehicle-treated mice compared with sham and rapamycin-treated mice (Figures 3C and 3D). In
Figure 2 Effects of rapamycin and the S6K inhibitor PF-4708671 on heart remodelling and function after MI

(A) Groups of mice, and the time course of rapamycin administration and the MI operation. Male mice (8 weeks of age) were divided into sham groups (I and II) and MI groups (III–VIII). Groups I and III were given vehicle. All of the other groups were given rapamycin at specific doses and for different time periods. Groups II and IV–VI were first given rapamycin for 1 week and then operated on (sham or MI), followed by another 4 weeks of rapamycin treatment. Group VII first underwent the MI procedure and were then given rapamycin for 4 weeks. Group VIII were similar to group VII, but were given rapamycin for only 1 week. At the end of the experiments, mice were examined for cardiac function by echocardiography and then killed for heart dissection and analysis. (B) Body weight of mice. Long-term treatment of rapamycin for 5 weeks slightly reduced body weight (groups II and IV–VI) as shown in Supplementary Figure S1(C) at http://www.BiochemJ.org/bj/441/bj4410199add.htm. Groups VII and VIII had similar body weights as group III. (C) The heart weight/body weight ratio. MI induced substantial heart remodelling (group III), but was significantly reduced by rapamycin treatment (groups IV–VII). Short-term treatment with rapamycin for 1 week did not reduce heart remodelling (group VIII). (D–I) Echocardiography measurements. Long-term treatment with rapamycin significantly improved heart function (indicated by EF and FS in D and E), and also reduced LV dilation (F). Treatment with PF-4708671 significantly improved heart function (indicated by EF and FS in G and H) and also reduced LV dilation (I). *P < 0.05 and **P < 0.01.

contrast, changes in cardiomyocyte structure and size were less in rapamycin-treated mice than vehicle-treated mice (Figures 3C and 3D).

We examined cardiomyocyte apoptosis using a TUNEL assay in both vehicle- and rapamycin-treated mice at 2 days after MI. The results indicated decreased apoptotic cardiomyocytes in rapamycin-treated mice compared with vehicle-treated mice (Figure 3E and Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410199add.htm). PF-4708671 administration also substantially reduced the fibrotic area, cardiomyocyte enlargement and apoptosis (Figures 3F–3I).

Taken together, these results indicate that S6K inhibition renders cardiac protection against MI.

Rapamycin and the S6K inhibitor PF-4708671 enhanced Akt Thr308 phosphorylation

In mammals, there are two mTORCs (mTOR complexes) termed mTORC1 and mTORC2 [6–8,29]. mTORC1 phosphorylates S6K at Thr389, whereas mTORC2 phosphorylates Akt at Ser473, leading to activation of these two kinases [5–7,30]. Long-term treatment with rapamycin inhibits both mTORC1 and mTORC2 which,
in turn, blocks S6K Thr^{389} and Akt Ser^{473} phosphorylation [31]. Accordingly, we detected effective inactivation of S6K and loss of Akt Ser^{473} phosphorylation in hearts after 7 days of treatment with rapamycin (Figures 4A and 4B, and Supplementary Figure S1A). Surprisingly, we found that Akt Thr^{389} phosphorylation was dramatically enhanced after 7 days of rapamycin treatment (Figures 4A and 4B). Next, we investigated these effects in mice following MI with 4 week of treatment with rapamycin. We dissected heart tissues from the infarct, border, and remote areas for Western blot analysis. First, we found that S6K activation...
was strongest in the infarct area, but its phosphorylation and that of Akt Ser\(^{473}\) were effectively blocked by rapamycin treatment. Secondly, Akt Thr\(^{308}\) phosphorylation was higher in all three areas in rapamycin-treated hearts than in controls (Figures 4C–4F). Furthermore, the phosphorylation of PRAS40, a new well-characterized Akt substrate, was increased (highest in the infarct area) compared with control, indicating that enhanced Akt Thr\(^{308}\) phosphorylation in the absence of Ser\(^{473}\) phosphorylation resulted in improved Akt activity (Figures 4C and 4G). Long-term treatment of rapamycin had no impact on ERK activation, but reduced p38 activation in hearts following an MI (Figures 4I and 4J).

PF-4708671 displayed similar effects as with rapamycin. PF-4708671 effectively inhibited S6K activation in the infarct, border and remote areas, and enhanced Akt Thr\(^{308}\) phosphorylation (Figure 5).

Deletion of PDK1 and Akt1/3 in cardiomyocytes prevented rapamycin-induced protection against MI

The results described above pointed to the possible cardiac-protective role of Akt Thr\(^{308}\) phosphorylation caused by rapamycin treatment. To test this, we investigated cardiac protection by rapamycin against MI in mice with the cardiomyocyte-specific deletion of PDK1, the upstream kinase of Akt Thr\(^{308}\) [32]. Because homozygous deletion of PDK1 in cardiomyocytes caused dilated cardiomyopathy and mortality at approximately 2 months of age in these mice [18,33,34], we analysed mice with a heterozygous deletion of PDK1. We observed decreased phosphorylation of both Thr\(^{308}\) and Ser\(^{473}\) of Akt in mice with half the cellular level of PDK1 absent in cardiomyocytes (Supplementary Figure S3).
S6K inhibition and myocardial infarction protection

Figure 6 Study of cardiomyocyte-specific PDK1-knockout mice after rapamycin treatment
(A–E) The MI procedure was performed in 8–12-week-old mice and the mice were treated with vehicle or rapamycin (rapa) for 4 weeks. At the end of the experiment, mice were examined by echocardiography and killed for heart dissection and analysis. (A) Heart weight/body weight ratio. (B) Comparison of the fibrotic area. (C–E) Echocardiographic parameters. (F) Western blot analysis. *P < 0.05. N.S, not significant; P, phospho-.

at http://www.BiochemJ.org/bj/441/bj4410199add.htm). Using a similar experimental procedure for the induction of MI, we failed to detect a protective effect after 5 weeks of treatment with rapamycin in these mice (Figures 6A–6E). In addition, we analysed the signalling pathways after MI and 4 weeks of rapamycin administration and were unable to detect an enhancement of Akt Thr308 phosphorylation in the heart (Figure 6F).

To confirm that Akt proteins play a major role in S6K-inhibition-mediated cardiac protection, we tested rapamycin in cardiomyocyte-specific Akt1/3-knockout mice after MI. However, we did not observe a protective effect after rapamycin treatment (Figures 7B–7F).

DISCUSSION

In the present study, we have demonstrated that inhibition of S6K with rapamycin and PF-4708671 effectively alleviated adverse remodelling following acute MI and significantly improved LV function in mice through enhancing Akt (at Thr308) signalling.

Previously, it has been reported that S6K activation plays a major role in heart hypertrophy and pathological remodelling, which can be suppressed by rapamycin in mouse models [15,35]. In the present study, we found rapid and substantial activation of S6K in the infarct area of the heart, suggesting that S6K also plays a major role in post-MI heart remodelling. Moreover, inhibition of S6K activation by rapamycin and PF-470-8671 suppressed post-MI heart remodelling and significantly improved LV function. Therefore these studies suggest that S6K activation in heart remodelling is provoked by distinct stimuli. In future studies, it will be necessary to investigate how S6K is activated by ischaemia or overload to induce pathological heart remodelling.

mTORC1 phosphorylates S6K at Thr389, whereas mTORC2 phosphorylates Akt at Ser473 [4–7]. Short-term treatment with rapamycin effectively inhibits mTORC1 and S6K activation without affecting mTORC2. It has been found that long-term treatment with rapamycin (more than 24 h) inhibited both mTORC1 and mTORC2 [31]. In the present study, we found that administration of rapamycin to mice for more than 1 week indeed suppressed S6K activation and Akt Ser473 phosphorylation, which is consistent with that found previously [31]. Moreover, we observed substantially enhanced Akt Thr308 phosphorylation induced by long-term rapamycin treatment, which in turn increased Akt activity because the phosphorylation levels of the Akt downstream target PRAS40 were induced. Previous work performed by Alessi and co-workers [36] has revealed a more important role of Akt Thr308 phosphorylation than Ser473 phosphorylation contributing to full Akt activation. They found that Akt activity is almost undetectable in the absence of Thr308 phosphorylation, although Ser473 phosphorylation is intact in PDK1-mutant ES (embryonic stem) cells [37]. The results of our present study indicate that enhanced Akt Thr308 phosphorylation alone could exert cardioprotection through promoting cardiomyocyte survival. Meanwhile, our results support the mechanism that S6K suppresses Akt (negative feedback) through PI3K (phosphoinositide 3-kinase), which promotes both Akt Thr308 and Ser473 phosphorylation [8,38]. Guertin and Sabatini [8] have observed a similar effect of Akt phosphorylation after mTOR inhibition using cell lines and they...
highlighted that Akt Thr308 phosphorylation was an indirect indicator of PI3K activation. Although it has been reported that rapamycin enhanced both Akt Thr308 and Ser473 phosphorylation in some tumour cell lines [39,40], our present work clearly shows that, in the heart, long-term administration of rapamycin effectively blocked Akt Ser473 phosphorylation, but increased Akt Thr308 phosphorylation.

PF-4708671 is a novel and highly specific S6K inhibitor [17]. In the present study, we have demonstrated that PF-4708671 could effectively suppress S6K activation in mouse heart tissue. Similar to rapamycin, PF-4708671 enhanced Akt phosphorylation, indicating that the negative regulation of Akt activity by S6K inhibition exists in mouse tissues. Furthermore, we found that PF-4708671 could reduce cardiomyocyte remodelling and reduce cardiomyocyte apoptosis, resulting in beneficial effects on cardiac protection against MI.

In some cell lines and patients, the antitumour growth property of rapamycin was not found to be sufficient enough because of increased Akt activity after rapamycin treatment, which became a serious side effect [40–43]. However, this side effect seems to be beneficial for heart function.

In summary, the cardiac protection of rapamycin and PF-4708671 against MI could be attributed to their effects on both the inhibition of S6K and the activation of Akt. On one hand, inhibition of S6K prevented adverse heart remodelling. On the other hand, enhanced Akt activity resulting from S6K inhibition promoted cardiomyocyte survival and heart function after MI [16,44,45]. Previous work performed by DeBosch et al. [46] uncovered the important role of Akt2 in cardiac protection using the Akt2-knockout mouse as a model [46]. They found that loss of Akt2 significantly increased infarct size compared with wild-type controls after MI. In the present study, we found that deletion of both Akt1 and Akt3 in cardiomyocytes severely impaired heart function after MI and administration of rapamycin had little effect in improving the situation. Taken together, these studies indicate that Akt signalling plays a critical role in protecting cardiac function following MI.

Rapamycin has been an approved clinical drug for many years, whereas PF-4708671 is a highly specific S6K inhibitor. Our present study suggests the potential novel function of rapamycin and PF-4708671 to treat patients following an MI and improve their heart function [47].

AUTHOR CONTRIBUTION
Ruomin Di and Xiangqi Wu designed the experiments and carried out most of the experimental work. Zai Chang generated the Akt1 floxed mice, and also the mice with cardiomyocyte-specific deletion of Akt1 and germline deletion of Akt3 (AktF/−; αMHC-Cre, Akt3−/−). Xia Zhao and Shuanghuang Lu provided technical advice and help. Quiting Feng maintained the mouse colony of cardiomyocyte-specific deletion of PDK1 (PDK1F/−; αMHC-Cre). Qing Luan contributed to the experimental design and provided technical advice. Brian Hemmings provided the Akt3−/− mice. Xinli Li and Zhongzhuo Yang directed the study and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt

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Figure S1 Effects of rapamycin on S6K inhibition and cardiac function

(A) Male mice (8 weeks of age) were treated with rapamycin (Rapa) at a dose of 10 mg/kg of body weight per day for 1 week and hearts were dissected for Western blot analysis. Four hearts were analysed for each group (control and rapamycin treatment). (B) Quantification of immunoblots in (A). (C–E) Male mice (8 weeks of age) were given rapamycin (10 mg/kg of body weight per day) for 10 weeks and the body weight, heart weight and function were measured. Long-term use of rapamycin slightly reduced the body weight of the mice (C). Heart weight was proportionally reduced along with body weight as the heart weight/body weight ratio was not changed after rapamycin treatment (D). Furthermore, rapamycin was not detrimental to heart function after long-term use as the EF was comparable between vehicle- and rapamycin-treated groups (E). *P < 0.05; N.S., not significant. P, phospho-.

Figure S2 TUNEL assay

Male mice (8 weeks of age) were treated with vehicle or rapamycin for 2 days after MI and hearts were dissected for TUNNEL assay. Apoptotic cardiomyocytes are red (To-pro-3 is an optimal fluorescent dye for nuclear counterstaining). Sections were taken from the border zone.

Figure S3 Western blot analysis of PDK1, Akt and phospho-Akt in hearts from heterozygous PDK1-knockout mice

For each group, three hearts from 8-week-old male mice were analysed. P, phospho-.

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