Pivotal Role of mTORC2 and Involvement of Ribosomal Protein S6 in Cardioprotective Signaling

Toshiyuki Yano, Marcella Ferlito, Angel Aponte, Atsushi Kuno, Tetsuji Miura, Elizabeth Murphy, Charles Steenbergen

Rationale: There is tight coupling between Akt activation and suppression of cell death. Full Akt activation requires mammalian target of rapamycin complex 2 (mTORC2), but the regulation of mTORC2 is unclear.

Objective: To gain new insights into mechanisms of mTORC2/Akt signaling.

Methods and Results: The role of mTORC2 in cardioprotection was examined. In perfused mouse hearts, ischemic preconditioning increased mTORC2 activity, leading to phosphorylation of Akt on Ser473. The protective effect of ischemic preconditioning was lost by pretreatment with dual mTORC inhibitors but not with rapamycin, an mTORC1 inhibitor, which indicates the fundamental role of mTORC2 activation in cardioprotection. Next, the regulation and downstream targets of mTORC2/Akt signaling were explored. We have found that ischemic preconditioning and other Akt activators (insulin and opioids) result in phosphorylation of ribosomal protein S6 (Rps6) at Ser235/236 in mouse hearts and neonatal rat ventricular myocytes. Rps6 interacted with components of mTORC2, and siRNA-mediated knockdown of Rps6 attenuates insulin-induced mTORC2 activation and Akt-Ser473 phosphorylation. On the other hand, Rps6 overexpression enhanced Akt-Ser473 phosphorylation, indicating that Rps6 activation amplifies mTORC2/Akt signaling. Disruption of the Rps6/mTORC2 pathway by knockdown of Rps6 or rictor abrogated insulin-induced cytoprotection against oxidative stress. Although rapamycin blocks Rps6-dependent mTORC2 activation, mTORC2 is still activated by an alternative signaling pathway, demonstrating the redundancy in cardioprotective signaling.

Conclusions: Activation of mTORC2 plays a pivotal role in cardioprotection, and Rps6 is a convergence point of cardioprotective signaling, providing positive feedback regulation of mTORC2/Akt signaling. (Circ Res. 2014;114:1268-1280.)

Key Words: insulin ■ ischemia-reperfusion injury ■ ischemic preconditioning ■ mTORC2 ■ myocardial ischemic reperfusion injury ■ rapamycin ■ sirolimus

Reperfusion therapy has improved the prognosis of patients with acute myocardial infarction, but it is insufficient in 25% patients, who have poor prognosis. Therefore, there is a need for adjunctive therapy in addition to reperfusion to improve clinical outcome. Several signaling pathways induced by cardioprotective interventions, such as ischemic preconditioning (IPC), ischemic postconditioning, and their mimetics, have been demonstrated. However, how these signaling pathways confer protection from cell death and how these pathways are amplified are not well understood.

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Phosphatidylinositol-3-kinase (PI3K)/Akt signaling is a major branch in cytoprotective signaling. Interventions that activate the PI3K/Akt pathway, such as IPC, insulin, erythropoietin, δ-opioid receptor agonists, and ischemic postconditioning, protect the heart from ischemia/reperfusion injury. The protective effect of IPC is abolished by PI3K inhibitors and in PI3K knockout mice or mice with a catalytically inactive mutant PI3K. Full activation of Akt requires phosphorylation at Thr308 by phosphoinositide-dependent kinase-1 (PDK1) and Ser473 by mammalian target of rapamycin complex 2 (mTORC2). Using PDK1 hypomorphic mutant mice with reduced expression of PDK1, a previous study showed that PDK1 was essential for IPC in perfused mouse hearts. However, there are no available data about the role of mTORC2 on IPC-induced protection. A recent thorough study by Miyamoto et al demonstrates that enhancement of Akt phosphorylation at Ser473, by PH domain leucine-rich repeat protein phosphatase-1 (PHLPP-1) deletion, suppresses cell death and ischemia/reperfusion injury, which suggests that mTORC2 is involved in cardioprotection.

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Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DADLE</td>
<td>[α-Ala2, α-Leu5]-enkephalin</td>
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<tr>
<td>GSK</td>
<td>glycogen synthase kinase 3</td>
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<tr>
<td>HEK293</td>
<td>human embryonic kidney cells</td>
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<tr>
<td>IPC</td>
<td>ischemic preconditioning</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>mTORC2</td>
<td>mTOR complex 2</td>
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<td>NRVM</td>
<td>neonatal rat ventricular myocytes</td>
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<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase-1</td>
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<tr>
<td>PHPP-1</td>
<td>PH domain leucine-rich repeat protein phosphatase-1</td>
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<tr>
<td>P13K</td>
<td>phosphatidylinositol-3-kinase</td>
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<tr>
<td>RPP</td>
<td>rate-pressure product</td>
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<td>Rps6</td>
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There is evidence of tight coupling between activation of PI3K/Akt signaling and suppression of mitochondrial permeability transition pore opening, which triggers cell death. First, multiple prosurvival signaling pathways, including Akt, converge on glycogen synthase kinase 3 (GSK3)-β, and inhibition of GSK3β suppresses opening of the mitochondrial permeability transition pore after reperfusion and ATP hydrolysis during ischemia. Second, Akt/endothelial nitric oxide synthase/nitric oxide signaling induces S-nitrosylation of proteins, which is proposed to reduce ischemia/reperfusion injury and increase the threshold for mitochondrial permeability transition pore opening. Finally, treatment with leukemia inhibitory factor suppresses mitochondrial permeability transition pore opening through Akt-mediated binding of hexokinase II with mitochondria, which is implicated in the protective mechanism of IPC.

In the present study, we characterized the regulation of mTORC2 by cardioprotective interventions and obtained new insights into the mechanisms by which Akt signaling suppresses cell death. We demonstrate that mTORC2 plays a crucial role in cardioprotection. Furthermore, ribosomal protein S6 (Rps6) is identified as a downstream target of the PI3K/Akt/mTOR signaling cascade. Interestingly, Rps6 regulates prosurvival ribosomal mTORC2 signaling, which serves as a positive feedback loop in Akt activation. Akt signaling can have both beneficial and detrimental effects, cardioprotection with acute activation, hypertrophy with more chronic activation. Better understanding of the role of this signaling in cardioprotection will provide opportunities for the development of novel cardioprotective interventions, with fewer detrimental effects.

Methods

Detailed Methods are in the Online Data Supplement.

Animals

This study was conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996) and approved by the Institutional Laboratory Animal Care and Use Committee. Male C57BL/6 mice (11–15 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Culture

Neonatal rat ventricular myocytes (NRVM) were isolated as described previously. Human embryonic kidney cells (HEK293) were obtained from ATCC.

Perfusion Protocols

Hearts were perfused as previously reported, and IPC was 4 cycles of 5-minute ischemia and 5-minute reperfusion. Ischemia/reperfusion injury was induced by 20-minute global ischemia, with 120-minute reperfusion for infarct measurement.

Immunoblotting and Immunoprecipitation

Samples for electrophoresis were total tissue homogenates or mitochondrial fractions prepared by differential centrifugation as previously reported.

mTORC2 Activity

We used a method reported by Huang with slight modification.

Results

IPC Activates mTORC2

We studied the role of mTOR in IPC-induced phosphorylation of proteins involved in cardioprotection, using the protocols in Figure 1A. The effect of different inhibitors was assessed on several key signaling molecules. IPC significantly increased phosphorylation of Akt-Ser473, Akt-Thr308, GSK3β, and endothelial nitric synthase, p70S6K, and Rps6 in mouse heart (Figure 1B and 1C). To explore the role of mTORC2 on Akt-Ser473 phosphorylation further, we measured mTORC2 activity. We immunoprecipitated mTORC2 using an antibody against rictor, and recombinant Akt was used as substrate. IPC increased mTORC2 activity by 1.8-fold (Figure 1D). When preconditioning was performed in the presence of wortmannin or Ku63794, mTORC2 activity was markedly reduced, as indicated by less phosphorylation of recombinant Akt on Ser473. A recent study showed that 1xβ kinase ε (IKKε) can directly phosphorylate Akt on Ser473 in a PI3K-dependent manner. IPC enhanced the ability of immunoprecipitated IKKε to phosphorylate Akt-Ser473 (Online Figure III). However, Ku63794 did not prevent IKKε activation by IPC (Online Figure III) but blocked phosphorylation of Akt-Ser473 by IPC, indicating the importance of mTORC2 in IPC. Thus, because mTORC2 is responsible for Ser473 phosphorylation and Rps6 is a downstream target of the Akt/mTORC1/p70S6K pathway, our results suggest that both mTORC1 and mTORC2 are involved in IPC-induced phosphorylation of key molecules involved in cardioprotection.

Cardioprotection Afforded by IPC Is Mediated by PI3K/mTORC2 Activation

We next evaluated the effect of PI3K/mTORC2 inhibition on IPC-mediated cardioprotection. As shown in Figure 2, 4 cycles of IPC limited infarct size from 49.6±3.1% to 15.9±2.8% and improved rate-pressure product (RPP) recovery from 42.7±6.0% to 74.1±4.6%. This protective effect of IPC on infarct size and RPP recovery was blocked by wortmannin (infarct size, 38.4±2.7%; RPP recovery, 42.3±4.2%) or the mTOR inhibitors Ku63794 (infarct size, 37.5±3.1%; RPP recovery, 55.4±4.3%) and pp242 (infarct size, 32.5±4.6%; RPP recovery, 55.0±2.5%). In contrast to the effect of dual mTOR inhibitors, IPC afforded similar cardioprotection in the presence of wortmannin or Ku63794.
inhibitors, infusion of rapamycin, an mTORC1 inhibitor, did not modify the effect of IPC on infarct size (21.3±2.7%) and RPP recovery (73.1±5.0%). These findings suggest that mTORC2 activation is involved in the mechanism by which acute IPC reduces ischemia/reperfusion injury, and mTORC1 activation is not required.

Cardioprotective Signals Mediated by the PI3K/Akt Pathway Converge on Rps6

Akt activation is important in cardioprotective signaling, and we sought to identify new possible downstream targets in the PI3K/Akt/mTOR signaling cascade. We initially screened for downstream proteins that were modulated by cardioprotective
interventions, using an antibody that detects phospho-Ser/Thr on the conserved motif RXRXX(S*/T*), where X is any amino acid, R is Arg (or Lys), and S*/T* denotes phosphorylatable serine/threonine residues. Several kinases can phosphorylate this motif (Figure 3D), including Akt, PKC, PKA, PKG, and the 70-kDa protein S6 kinase (p70S6K). First, the effects of IPC and insulin, PI3K/Akt activators, were examined. Among the proteins that showed increased phosphorylation by IPC or insulin was a 32-kDa protein (Figure 3A). Surprisingly, 100 μmol/L of diazoxide, another well-known IPC mimetic, also increased phosphorylation of this 32-kDa protein (Figure 3A). In NRVM, wortmannin and LY294004, PI3K inhibitors, reduced phosphorylation of this 32-kDa protein (Figure 3B). Because the 70-kDa protein S6 kinase (p70S6K) favors substrates that have the conserved motif RXRXX(S*/T*) and previous studies identified a 30- to 32-kDa protein that is phosphorylated by receptor agonists that activate PI3K in cell culture as Rps6,20,21 we examined the effect of mTOR inhibitors on phosphorylation of this 32-kDa protein. Phosphorylation was reduced by rapamycin, an mTORC1 inhibitor, and Ku63794, an ATP competitive dual mTOR inhibitor (Figure 3B), indicating that phosphorylation of this 32-kDa protein is regulated by the mTORC1/p70S6K pathway (Figure 3B).

To corroborate our hypothesis that this 32-kDa protein is Rps6, we performed Western blots with an antibody that detects phosphorylation of Rps6 on Ser235 or Ser236 and found that the bands for the 32-kDa phosphoprotein identified by the phospho-RXRXX(S*/T*) antibody and by the p-Rps6 antibody migrated in exactly the same location on the gel and the intensity changed in parallel (Figure 3B; Online Figure IV). We transfected HA-tagged Rps6S235A/S236A with Ser replaced by Ala at 235 and 236 on Rps6, and its control (Rps6WT) in HEK293 cells. Rps6WT but not Rps6S235A/S236A was clearly detected by immunoblotting with the phospho-RXRXX(S*/T*) antibody (Online Figure IV). Furthermore, we used siRNA against Rps6 and observed a decrease in the 32-kDa phosphoprotein that increases with cardioprotective interventions (Figure 3C). Stimulation with insulin, [d-Ala2, d-Leu5]-enkephalin (DADLE), 8-bromoadenosine 3′,5′-cyclic monophosphate, DOG (1,2-dioctanoyl-sn-glycerol), diazoxide, and 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate all increased phosphorylation of Rps6 at Ser235 or Ser236 (Figure 3E; Online Figure V). Previous studies showed that Akt phosphorylation is reduced during ischemia and increased after reperfusion.22 We found that in perfused mouse hearts, the pattern of Rps6 phosphorylation during ischemia/reperfusion was also similar to Akt phosphorylation and GSK3β phosphorylation, a direct Akt substrate (Online Figure VI). IPC alone significantly increased phosphorylation of Rps6, and a significant difference between control and IPC hearts was maintained during ischemia and after reperfusion (Online Figure VI). Taken together, the data indicate that Rps6 phosphorylation is a convergence point of multiple cardioprotective signals.

Finally, we examined whether Akt can directly phosphorylate Rps6. In vitro analysis showed that active Akt could not phosphorylate Rps6 immunoprecipitated from

**Figure 2. Cardioprotection afforded by ischemic preconditioning (IPC) is mediated by mammalian target of rapamycin complex 2 (mTORC2).**

**A.** The effect of inhibitors on infarct size. Infarct size is expressed as a percentage of ventricular area. **B.** Postischemic rate-pressure product (RPP) recovery. RPP recovery after reperfusion is shown as a percentage of preischemic RPP after stabilization. Black bar, control; gray bar, IPC. n=5 to 7 in each group. *P<0.05 vs control. †P<0.05 vs IPC, no inhibitor.
Rps6-overexpressed HEK293 cells (Online Figure VII). Thus, phosphorylation of Rps6 by IPC and insulin is primarily regulated by the Akt/mTORC1/p70S6K pathway, but further analysis is needed to assess whether Akt can directly phosphorylate Rps6 in vivo.

**Rps6 Is Associated With mTORC2 and Regulates Prosurvival mTORC2/Akt Signaling**

The primary role of Rps6 is protein translation, but the involvement of protein translation in the protective mechanisms of classical/early IPC is controversial. A recent study demonstrated that insulin induces the association of ribosomes with mTORC2 and enhances mTORC2 activity in a protein translation-independent mechanism. However, how insulin activates the mTORC2–ribosome complex remains unclear. We sought to determine whether Rps6 phosphorylation induced by protective signaling is a key step to mTORC2 activation.

First, the association of Rps6 with mTORC2 was examined. mTOR exists in 2 different complexes. mTOR interacts with raptor to form an mTORC1 and with rictor to form an mTORC2. To immunoprecipitate mTORC2, anti-rictor antibody was used. In mouse hearts, phospho-Rps6 was coimmunoprecipitated with mTORC2 under basal conditions, and the amount of phospho-Rps6 bound to mTORC2 was increased by IPC (Figure 4A). IPC did not modulate the levels of mTOR and Rps6 in the rictor immunoprecipitates (Figure 4A). Insulin treatment in HEK293 cells also increased the levels of phospho-Rps6 but not of Rps6 bound to mTORC2, which was blocked by pretreatment with the PI3K inhibitor LY294004 (Figure 4B). To examine whether Rps6 bound to mTORC2 in the ribosomal protein complex is phosphorylated, immunoprecipitation using the antibody against ribosomal protein L26, a protein in the 60S ribosomal complex, showed that the association of Rps6 with mTORC2 is increased by IPC.
Insulin increased phosphorylation of Rps6 in the homogenate and the amount of p-Rps6 in the ribosomal complex although mTORC2 levels in the ribosomal complex were not modulated by insulin (Online Figure VIII). These findings suggest that Akt signaling increases the amount of phospho-Rps6 that is bound to mTORC2 in the ribosomal complex.

Next, we examined the role of Rps6 in mTORC2 signaling. mTORC2 is the kinase that phosphorylates Akt on Ser473. We hypothesized that activation of PI3K/Akt signaling induces Rps6 phosphorylation, which in turn leads to phosphorylation of Ser473 through mTORC2 activation. In this way, Rps6 serves as a positive feedback loop in Akt signaling (Figure 4C). We investigated the importance of Rps6 phosphorylation in signaling and in cytoprotection.

To study the role of Rps6 phosphorylation in these signaling pathways, the level of Rps6 phosphorylation was reduced by siRNA-mediated knockdown of Rps6 and by mTOR inhibitors. Insulin induced significant phosphorylation of Akt, GSK3β, and Rps6 in serum-free NRVM and HEK293 cells. The level of Akt-Ser473 phosphorylation was increased at 5 minutes after insulin addition and then declined (Figure 5A...
Ribosomal protein S6 (Rps6) is associated with mammalian target of rapamycin complex 2 (mTORC2). A and B. The effect of ischemic preconditioning (IPC; A) or insulin (B) on interaction of mTORC2 with Rps6 in mouse hearts (A) or human embryonic kidney (HEK293) cells (B), respectively. mTORC2 was immunoprecipitated with anti-rictor antibody from mouse heart treated with or without IPC. Representative immunoblots and summarized data are presented. *P<0.05 vs control. n=3 in each group. C, Schema of protective signaling by IPC and insulin, highlighting the stimulatory effect of Rps6 phosphorylation on mTORC2 (red line). GPCR indicates G-protein–coupled receptor; IP , immunoprecipitation; PDK, phosphoinositide-dependent kinase; and PI3K, phosphatidylinositol-3-kinase.

and 5B). However, the level of Rps6 phosphorylation was gradually augmented and sustained. Knockdown of Rps6 in NRVM significantly reduced phosphorylation of Akt-Ser473 and GSK3β, but not Akt-Thr308 phosphorylation, after 15 minutes of insulin stimulation when compared with control siRNA (Figure 5B). A similar trend in the effect of Rps6 knockdown on Akt/GSK3β signaling was observed in HEK293 cells (Figure 5A). Next, we examined the effect of pp242, an mTOR inhibitor, on phosphorylation of Akt and Rps6. Unlike the effect of Rps6 knockdown, phosphorylation of Akt-Ser473 was attenuated by pp242 even at 5 minutes after insulin addition (Online Figure IX). Thus, Rps6 is critically involved in the sustained mTORC2-mediated Akt-Ser473 phosphorylation and downstream effects of Akt signaling, but an Rps6-independent mechanism also contributes to phosphorylation of Akt-Ser473 by insulin.

Although PI3Kα is involved in Akt activation by insulin, PI3Kγ mediates Akt phosphorylation through G-protein–coupled receptors, such as the δ-opioid receptor. To assess whether Rps6 is involved in the signaling pathway induced by DADLE, a δ-opioid receptor agonist, we examined the effect of Rps6 knockdown on DADLE signaling. Rps6 knockdown attenuated phosphorylation of Akt-ser473 and GSK3β after 15 minutes of DADLE treatment (Figure 5C). Rps6 downregulation also decreased insulin-induced mTORC2 activity, assessed by an in vitro kinase assay of Akt phosphorylation in HEK293 cells (Figure 5D) and NRVM (data not shown). Conversely, overexpression of HA-Rps6 increased phosphorylation of Akt-ser473 under nonstimulated conditions, resulting in enhancement of endogenous Rps6 phosphorylation (Online Figure X). These findings demonstrate that Rps6 regulates mTORC2 activity induced by PI3K activation and mediates positive feedback signaling in mTORC2/Akt activation.

Finally, the role of mTORC2 and Rps6 in cytoprotection afforded by insulin was examined in NRVM. Incubation with 100 μmol/L H2O2 for 18 hours induced cell death in ≈30% of NRVM. Pretreatment with insulin significantly suppressed cell death by 70% when compared with no pretreatment controls (Figure 6). mTOR inhibition by Ku63794 blocked cytoprotection induced by insulin, indicating that this experimental model simulated IPC-induced cardioprotection in perfused hearts. Consistent with our hypothesis, knockdown of rictor (Online Figure XI) or Rps6 abolished the protection afforded by insulin. Thus, mTORC2 and Rps6 are critical for the protective effect induced by insulin.

Because it is possible that Rps6 knockdown itself could inhibit overall protein synthesis and induce cell injury, the effect of Rps6 knockdown on confounding factors in these experimental results was examined. Protein levels of mTOR, rictor, Akt, GSK3β, ribosomal protein L26, and PHLP-1, a phosphatase that dephosphorylates p-Akt at ser473, were comparable between NRVM transfected
with control siRNA and Rps6 siRNA (Online Figure XII). Although deficiency of ribosomal proteins, including Rps6, can induce p53-dependent cell cycle arrest and apoptosis,24 p53 was not induced by Rps6 knockdown in our experimental protocol (Online Figure XIII). Thus, our protocol, which reduced Rps6 by ≈50%, had no detectable effect on protein levels or cell injury.

**Reduction of Rps6 Phosphorylation by Rapamycin Reduced Ribosomal mTORC2 Activity but Did Not Eliminate the IPC-Induced Overall Increase in mTORC2 Activity**

Rapamycin reduces Rps6 phosphorylation through mTORC1 inhibition, which should reduce mTORC2 activation according to our Rps6 knockdown data. Phosphorylation of Rps6 by IPC

**Figure 5. Ribosomal protein S6 (Rps6) regulates mammalian target of rapamycin complex 2 (mTORC2)/Akt signaling.**

A, Representative immunoblots showing the effect of siRNA-mediated knockdown of Rps6 on insulin-induced Akt/glycogen synthase kinase 3 (GSK3)-β signaling in neonatal rat ventricular myocytes (NRVM) and human embryonic kidney (HEK293) cells. Insulin (200 nmol/L) treatment was performed at 24 hours (HEK293 cells) or 40 hours (NRVM) after siRNA transfection. B, Summarized data from insulin-induced Akt/GSK3-β signaling in NRVM. Levels of phosphorylated kinases were normalized to α-tubulin levels. Black bar, control siRNA transfection; gray bar, Rps6 siRNA transfection. n=4 in each group. *P<0.05 vs control. C, The effect of siRNA-mediated knockdown of Rps6 on [d-Ala2, d-Leu5]-enkephalin (DADLE)–induced Akt/GSK3-β signaling in NRVM. DADLE (300 nmol/L) treatment was performed at 40 hours after siRNA transfection. Representative blots are shown. D, The effect of siRNA-mediated knockdown of Rps6 on insulin-induced mTORC2 activation in HEK293 cells. Insulin stimulation was performed at 24 hours after siRNA transfection. Rictor immunoprecipitates from whole cell homogenates were prepared for in vitro kinase assay. Three independent experiments showed similar results.
was significantly reduced by rapamycin (Figure 7A and 7B). Nevertheless, rapamycin did not affect the IPC-induced increase in Akt phosphorylation at Thr308 or Ser473 (Figure 7A and 7B). In addition, rapamycin treatment failed to reduce the increase in mTORC2 activity by IPC (Figure 7C). It is well known that the PI3K/Akt pathway is upregulated by mTORC1 inhibition because of rapamycin treatment through disruption of negative feedback control of PI3K/Akt signaling.25 We hypothesize that rapamycin reduces the activity of mTORC2 bound to ribosomes but enhances ribosome-independent mTORC2 activation (Online Figure XIV). To evaluate ribosomal mTORC2 activity, we immunoprecipitated mTORC2 using an antibody against ribosomal protein L26 (Online Figure VIII). In contrast to total mTORC2 activity, measured using mTORC2 immunoprecipitated with rictor antibody, IPC-induced ribosomal mTORC2 activity was reduced by rapamycin (Figure 7D). These data suggest that enhancement of mTORC2 activity by IPC is maintained by the activation of mTORC2 through a ribosome-independent mechanism, despite a decrease in ribosomal mTORC2 activation through reduction of Rps6 phosphorylation (Online Figure XIV).

Discussion
This is the first study to examine specifically the role of mTORC2 in cardioprotection. The novel findings obtained from this study are as follows: (1) mTORC2 activation plays a pivotal role in the cardioprotective mechanisms of IPC, including phosphorylation of GSK3β and endothelial nitric synthase. (2) Rps6 was identified as a downstream target of PI3K/Akt pathway and multiple cytoprotective signals phosphorylated Rps6 at Ser235/236. (3) Direct reduction of Rps6 phosphorylation by Rps6 knockdown attenuated insulin- and
opioid-induced mTORC2 activation and Akt-Ser473 phosphorylation. (4) Pharmacological and siRNA-mediated disruption of the Rps6/mTORC2 pathway eliminated the protective effect of insulin-induced pharmacological preconditioning. (5) Upregulation of Rps6 increased phosphorylation of Akt-Ser473. (6) Reduction of Rps6 phosphorylation by rapamycin also decreased ribosomal mTORC2 activity, but IPC-induced mTORC2 activation and Akt-Ser473 phosphorylation persisted, through stimulation of nonribosomal mTORC2 activity. These data show the essential role of mTORC2 activation in cytoprotection induced by PI3K/Akt signaling and the importance of Rps6 phosphorylation in mTORC2 activation.

**Pivotal Role of mTORC2 in Cardioprotective Signaling**

Cardioprotection requires activation of Akt. Full activation of Akt requires phosphorylation at Thr308 and Ser473. The literature suggests that only PDK1 phosphorylates Akt at Thr308 in the activation loop, whereas mTORC2 is the primary Akt-Ser473 kinase although this is not the sole mechanism of Akt phosphorylation at Ser473. Using PDK1 hypomorphic mutant mice with reduced expression of PDK1, a previous study showed that PDK1 was essential for IPC in perfused mouse hearts. However, there are no available data about the role of mTORC2 on IPC-induced protection. In our report, the infarct size limitation afforded by IPC was blocked by wortmannin, a PI3K inhibitor, and ATP competitive dual mTOR inhibitors, which inhibit both mTORC1 and mTORC2 activity but not rapamycin, an mTORC1 inhibitor. IPC-induced mTORC2 activation and Akt-Ser473 phosphorylation were blocked by wortmannin and dual mTOR inhibitors but not rapamycin. Although mTOR inhibition can attenuate IPC-induced Akt-Thr308 phosphorylation, the decrease in Akt-Thr308 phosphorylation by mTOR inhibitors was significantly less than by wortmannin, indicating that mTOR inhibitors did not completely inhibit P3K or PDK1 activity. Finally, constitutive phosphorylation of Akt-Ser473 by knockout of PHLP-1, an Akt-Ser473–specific
Involvement of Rps6 in Cardioprotective Signaling

Rps6 is a component of the small 40S ribosomal subunit. The fundamental role of ribosomes is protein translation, but some protein translation-independent functions have been reported. A recent study by Zinzalla et al. demonstrated that ribosomes are involved in insulin-induced mTORC2 activation in cancer cell lines. In that study, knockdown of 3 different ribosomal proteins decreased levels of other ribosomal proteins, indicating impaired ribosomal assembly. Under those conditions, H_{2}O_{2}-induced apoptosis was higher and insulin-induced mTORC2 activation was reduced. Further analysis suggested that increased association of mTORC2 with ribosomes by insulin is the mechanism responsible for insulin-induced mTORC2 activation. In our report, IPC and insulin did not affect the level of association of mTORC2 with ribosomes but enhanced phosphorylation of Rps6 in the ribosome/mTORC2 complex (Figure 4). Rps6 knockdown inhibited mTORC2 activation by insulin and DADLE (Figure 5), and insulin-induced cytoprotection was lost by Rps6 knockdown (Figure 6). Thus, Rps6 is a primary mediator in the cytoprotection that occurs when the PI3K/mTORC2 pathway is activated by pharmacological agents, such as insulin, in cardiomyocytes and mouse hearts.

The involvement of protein translation in the mechanism of IPC is controversial. We have found that cardioprotective interventions modulate subcellular protein levels by post-translational modification and regulation of mitochondrial import and degradation, thus, in a protein translation-independent manner, an earlier study by the group of Downey showed that pharmacological inhibition of protein translation did not affect IPC-induced infarct size limitation. Furthermore, Zinzalla et al. reported that protein translation inhibitors did not affect phosphorylation of Akt-Ser473 by insulin. Thus, it seems that cardioprotection can be achieved independent of changes in protein translation.

Our data and the literature suggest that there is a complex connection between PI3K signaling and mTORC2 activation. A previous study using an in vitro kinase assay showed that immunoprecipitated mTORC2 was activated by phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which can explain prompt phosphorylation of Akt-Ser473 by insulin or by IPC. In the present study, the level of Akt-Ser473 phosphorylation was increased at 5 minutes after insulin addition and then declined. However, Rps6 phosphorylation was maintained at a high level after insulin treatment (Figure 5A and 5B). Furthermore, significant differences in Akt-Ser473 phosphorylation between control siRNA- and Rps6 siRNA-transfected cells were observed at 15 minutes but not at 5 minutes after insulin addition. In contrast, phosphorylation of Akt-Ser473 was attenuated by pp242 even at 5 minutes after insulin addition (Online Figure IX). These findings suggested that Rps6 phosphorylation contributed more to mTORC2 activation at 15 minutes after insulin addition than at earlier times, and that early activation of mTORC2 was induced by a different mechanism, presumably through direct activation by PIP3. Thus, there is Rps6-dependent and Rps6-independent mTORC2 activation, and Rps6-dependent mTORC2 activation serves as a positive feedback loop in Akt signaling (Online Figure XIV and XV). Similarly, with IPC, rapamycin blocks Rps6 phosphorylation but does not prevent Akt-Ser473 phosphorylation, which can occur by the Rps6-independent process. Because there is only a 5-minute gap between IPC and the sustained period of ischemia, the ability to sustain mTORC2 activation would be less important in our protocol of IPC than in some models of pharmacological preconditioning. Multiple negative feedback mechanisms in PI3K/Akt/mTOR signaling have been proposed and demonstrated, but there are little data on positive feedback mechanisms, including the Rps6-dependent pathway, before our study.

Our data suggest that Rps6 phosphorylation is important for mTORC2 activation through the mTORC1/Rps6-dependent pathway, particularly for sustained activation. Ruvsinsky et al. generated a knockin mouse (rps6^{Δ+Δ}) encoding a mutant Rps6 harboring Ala substitutions at all 5 C-terminal phosphorylation sites (Ser235, Ser236, Ser241, Ser244, and Ser247). In that study, comparable phosphorylation of Akt-Ser473 at 5 minutes after intraperitoneal injection of insulin was observed in rps6^{ΔΔ} when compared with that in wild-type mice. Although this early response to insulin in rps6^{ΔΔ} was similar to that in Rps6 siRNA-transfected NRVM (Figure 5A and 5B), the time course of Akt phosphorylation by insulin stimulation in rps6^{ΔΔ} was not examined. Furthermore, there is the possibility that other ribosomal proteins might also mediate this signaling, particularly in a chronic model of impaired Rps6 signaling. In addition to the study by Zinzalla et al., previous studies reported that Rps3 knockdown increased H_{2}O_{2}-induced cell death in HEK293 cells, and Rps3 was identified as an Akt substrate. Interestingly, our study showed that IPC induced S-nitrosylation of ribosomal proteins, including Rps6 in perfused mouse hearts. The role of other ribosomal proteins and S-nitrosylation of ribosomal proteins in regulation of mTORC2 warrants further investigation. Nevertheless, Rps6 is an important transducer of mTORC2 activation because our data show that Rps6 knockdown resulted in less phosphorylation of Akt-Ser473 at 15 minutes after insulin treatment, and Rps6 knockdown eliminated the cytoprotection induced by pharmacological preconditioning.

Effect of Rapamycin on Ischemia/Reperfusion Injury and Cardioprotection

Previous studies have shown that infarct size limitation afforded by IPC, ischemic postconditioning, and their mimetics
can be blocked by rapamycin in rat regional ischemia models and a mouse perfused heart model although there are conflicting results. Because p70S6K can directly phosphorylate GSK3β, rapamycin-reduced induction of GSK3β phosphorylation was proposed as a primary mechanism of the inhibitory effect of rapamycin on cardioprotection. In contrast, pretreatment with rapamycin alone reduced infarct size in mouse global ischemia models and protected from hypoxia/reoxygenation-induced cardiomyocyte death. In addition, our previous study showed that the infarct size-limiting effect of IPC was not necessarily correlated with the level of p70S6K phosphorylation. The effect of rapamycin on signaling downstream of PI3K is highly complex. Rapamycin reduces mTORC1 activity, which decreases phosphorylation of p70S6K and Rps6. Because a decrease in Rps6 phosphorylation by Rps6 knockdown reduced mTORC2 activity (Figure 5), it is possible that rapamycin could attenuate IPC-induced Akt phosphorylation and mTORC2 activation. However, it is well known that the PI3K/Akt pathway is upregulated by mTORC1 inhibition because of rapamycin treatment or raptor knockout through disruption of negative feedback control of PI3K/Akt signaling (Online Figure XIV). In our report, rapamycin did not affect the IPC-induced increase in Akt phosphorylation and overall mTORC2 activity although reduction of Rps6 phosphorylation by rapamycin decreased ribosomal mTORC2 activity (Figure 7). In addition, infarct size limitation afforded by multiple cycles of IPC was not blocked by rapamycin (Figure 2). A plausible explanation is that IPC activates prosurvival mTORC2 by 2 mechanisms: through a Rps6/ribosome-independent mechanism and through Rps6-dependent ribosomal mTORC2 activation, and only the latter is reduced by rapamycin (Online Figure XIV). It will be important to analyze the time-dependent changes mediated by rapamycin in signaling pathways responsible for cardioprotection in future studies.

Conclusions

IPC activates multiple classes of receptors, leading to the activation of redundant prosurvival signaling pathways. In particular, repetitive IPC augments activation of redundant signaling pathways so that blockade of a single signaling pathway in IPC does not abrogate protection because of compensation by other signaling pathways. The activation of multiple signaling pathways strengthens the protective effect. We find that mTORC2 plays a key role in cardioprotection through phosphorylation of Akt-Ser473, which results in full Akt activation. mTORC2 can be activated by a ribosomal Rps6-dependent pathway and by a nonribosomal Rps6-independent pathway. The ribosomal Rps6-dependent pathway confers sustained Akt activation that could be more useful for cardioprotection. Because multiple cardioprotective signaling pathways converge on mTORC2 and phosphorylation of Rps6, Rps6/mTORC2 signaling may be an attractive target for the development of novel cardioprotective interventions.

Acknowledgments

We thank Dr Roux for kindly providing ribosomal protein S6 plasmids.

Sources of Funding

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- Phosphatidylinositol-3-kinase/Akt activation initiates multiple pathways of cytoprotective signaling induced by agonists of G-protein–coupled receptors and ischemic preconditioning.
- Full Akt activation requires dual phosphorylation, and mammalian target of rapamycin complex 2 (mTORC2) has been shown to phosphorylate Akt on Ser473.
- There is tight coupling between Akt activation and suppression of cell death.

What New Information Does This Article Contribute?

- mTORC2 activation plays a pivotal role in cardioprotection.
- Ribosomal protein S6 (Rps6) was identified as a downstream target of phosphatidylinositol-3-kinase/Akt/p70S6K signaling and multiple cytoprotective signals increase phosphorylation of Rps6 on Ser235/236.
- Phosphorylation of Rps6 by the phosphatidylinositol-3-kinase/Akt/p70S6K pathway increases mTORC2 activity, leading to enhanced and longer-lived phosphorylation of Akt on Ser473.
- The Rps6/mTORC2 signaling module is responsible for insulin-induced cytoprotection against oxidative stress.

Multiple cardioprotective signaling pathways have been identified, but the mechanisms by which these signaling pathways are amplified and how they confer protection from cell death are not well understood. We show that activation of mTORC2 plays a pivotal role in cardioprotection afforded by ischemic preconditioning and other cardioprotective interventions. We propose dual pathways of mTORC2 activation that are involved in cardioprotection. The activation of the phosphatidylinositol-3-kinase/Akt pathway, a well-known cardioprotective signal, leads to Rps6 phosphorylation-mediated mTORC2 activation. Thus, Akt activation begets further Akt activation through Rps6/mTORC2 signaling. The reduction of Rps6 phosphorylation by rapamycin also blocks Rps6-dependent mTORC2 activation, but mTORC2 is still activated by an alternative signaling pathway, demonstrating the redundancy in cardioprotective signaling. However, ribosomal Rps6-dependent mTORC2 activation results in greater and more persistent Akt activation. Better understanding of the role of the dual pathways of mTORC2 activation in cardioprotection will likely provide opportunities for the development of novel cardioprotective interventions.
Pivotal Role of mTORC2 and Involvement of Ribosomal Protein S6 in Cardioprotective Signaling
Toshiyuki Yano, Marcella Ferlito, Angel Aponte, Atsushi Kuno, Tetsuji Miura, Elizabeth Murphy and Charles Steenbergen

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SUPPLEMENTAL MATERIAL

The pivotal role of mTORC2 and involvement of ribosomal protein S6 in cardioprotective signaling

Yano T, Ferlito M, Aponte A, Kuno A, Miura T, Murphy E, and Steenbergen C

Detailed Methods

Animals: This study was conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Institutional Laboratory Animal Care and Use Committee. Male C57BL/6 mice (11 to 15 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Isolated heart preparation: Anesthetization and preparation of isolated heart were performed as described previously with slight modification (1, 2). In brief, mice were anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and hearts were quickly excised and perfused at a pressure of 100 cm with non-circulating Krebs-Henseleit buffer (NaCl 120, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 1.75 and glucose 10 mmol/L). The buffer was gassed with 95% O2/5% CO2, and the temperature of the perfusate was maintained at 37°C. A fluid-filled latex balloon was inserted into the left ventricle to monitor hemodynamics.

Ischemia/reperfusion protocol and infarct size measurement: After equilibrium perfusion of at least 20 minutes, followed by IPC (4 cycles of 5 min of ischemia and 5 min of reperfusion) or an additional 20 minutes of control perfusion, mouse hearts were subjected to 20 min of global ischemia, followed by 120 min of reperfusion for infarct size determination. Hearts were
assigned to one of five pretreatments before global ischemia: DMSO control (no inhibitor), infusion of wortmannin (200 nmol/L), infusion of Ku-0063794 (Ku63794, 1 μmol/L), infusion of pp242 (0.5 μmol/L) or infusion of rapamycin (1 nmol/L) as shown in the Figure 1A. The infusion of these inhibitors did not affect hemodynamic parameters (Data not shown). Left ventricular developed pressure (LVDP) was recorded and digitized using a power lab system (ADInstruments, Colorado Springs, CO). After 2 hours of reperfusion, hearts were perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated in TTC at 37°C for 15 min, followed by fixation in 10% formaldehyde. Infarct size was determined as the percentage of total ventricular area (1).

**Primary culture of neonatal rat ventricular myocytes:** Neonatal rat ventricular myocytes (NRVM) were isolated from the whole heart of 1-3 days old rats as described previously (3). In brief, the hearts were minced, digested with trypsin overnight at 4 °C. The day after, tissue was dissociated by stepwise collagenase treatment for a few minutes at 37 °C. Cells were pre-plated twice for 60 minutes to eliminate fibroblasts and enrich the culture for cardiac myocytes. The non-adherent myocytes were then plated at a density of 1200 cells/mm² in plating medium consisting of 199 medium supplemented with HEPES, MEM non-essential amino acids, glucose, glutamine, 10% FBS, vitamin B12, penicillin, streptomycin, on fibronectin coated plates. The next day cells were washed and fresh medium with 2% FBS was added. The cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator.

**Transfection:** To demonstrate the role of Rps6 on phosphorylation of Akt at Ser473 and mTORC2 activity, 50 nmol/L of Rps6 siRNA was transfected into NRVM using an electroporator (Nucleofector, Amaxa, Gaitherburg, MD) following the protocol for NRVM. We previously confirmed that transfection efficiency in this method, which was assessed by GFP expression using FACS analysis, was 62.0 ± 2.9% (3). Rps6 siRNA and non-silencing siRNA
were purchased from Ambion (Silencer Select, Austin, TX). In preliminary experiments, we tested 3 different types of siRNA for Rps6 and only s131129 (Sense: GCAGAAUGCUAAACUUUtt, Antisense: AAAGUUUAGCAUUCUGag) was clearly effective, consistent with a recent study (4). Rictor siRNA (ON-TARGETplus Rat Rictor) was purchased from Dharmacon. NRVM were stimulated with 200 nmol/L insulin or 300 µmol/L DADLE at 40 h after transfection with siRNA. NRVM were serum starved for 18 h before stimulation. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and stimulated at 24 h after transfection.

The HA-Rps6<sup>WT</sup> plasmid and the HA-Rps6<sup>S235A/S236A</sup> were kindly provided by Dr. Phillipe Roux, University of Montreal, and transfected into HEK293 cells using FuGENE HD (Promega, Madison, WI).

**Immunoblotting and immunoprecipitation:** To obtain total homogenate, frozen heart samples were homogenized in ice-cold buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na<sub>2</sub>EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor cocktail (Complete mini, Roche Molecular Biochemicals, Mannheim, Germany), and a phosphatase inhibitor cocktail (PhosSTOP, Roche Molecular Biochemicals). To preserve the integrity of mTORC2 in co-immunoprecipitation analysis, heart samples were homogenized in ice-cold CHAPS buffer containing 20 mmol/L HEPES (pH 7.5), 120 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L NaF, 0.3% CHAPS, 0.5 mmol/L Na<sub>3</sub>VO<sub>4</sub>, a protease inhibitor cocktail (Complete mini), and a phosphatase inhibitor cocktail (PhosSTOP). The homogenate was centrifuged at 13,000 g for 15 min to obtain the supernatant. Protein concentration was determined using Bradford assay.

Equal amounts of proteins were electrophoresed on 4~12% or 3~8% polyacrylamide
gels and then blotted onto PVDF membranes (Millipore, Bedford, MA). After blocking had been performed with a TBS-T buffer containing 5% nonfat dry milk or 5% BSA, the blots were incubated with antibodies that recognize the following: phospho-RXRXX(S*/T*), phospho-Rps6 (Ser235/236), Rps6, phospho-Akt (Ser473, Thr308) and total Akt, phospho-GSK3β (Ser9) and total GSK3β, mTOR, Rictor, p53 1C12 (Cell Signaling Technology, Beverly, MA); Rps6, Rpl26, PHLPP-1 (Bethyl Laboratories, Montgomery, TX); HA (Millipore, Billerica, MA); and α−tubulin (Abcam, Cambridge, MA). We used the mixture of both anti-mTOR antibody and anti-rictor antibody to detect both mTOR and rictor simultaneously. Immunoblotted proteins were visualized by using an Immobilon Western detection kit (Millipore, Billerica, MA).

Immunoprecipitation was performed as described previously with modification (5, 6). Protein G magnetic beads (Dynabeads, Invitrogen, Carlsbad, CA) were washed and resuspended in CHAPS buffer. Anti-rictor antibody, anti-Rpl26 antibody, and rabbit IgG were added and incubated at room temperature with gentle agitation for 30 min to form antibody-beads complex. Five hundred μg ~ 1000 μg of homogenates were pre-incubated with 40 μl of magnetic beads for 1 h (pre-cleaned). After the beads had been discarded, the pre-cleaned supernatant was incubated with antibody-beads complex for 2 h. A magnetic field was applied to this IP mixture, and the supernatant was removed. The beads were washed 4 times using 500 μl of CHAPS buffer, re-suspended in 40 μl of LDS sample loading buffer (Invitrogen), and incubated at 70 °C for 10 min. The supernatant was used for immunoblotting.

**In vitro kinase assay:** To prepare mTORC2 and IKKε immunoprecipitates, immunoprecipitation was performed as described above. Anti-rictor or anti-IKKε antibody-bead complex was incubated with pre-cleaned 500 μg of homogenates for 2 h. After the supernatant was removed, the beads were washed 3 times with 500 μl of CHAPS buffer and then with kinase assay buffer containing 25 mmol/L HEPES (pH 7.4), 100 mmol/L sodium acetate, 1
5 mmol/L magnesium chloride. The beads with immunoprecipitated mTORC2 or IKKe were incubated with 0.5 µg of recombinant Akt (Millipore, Bedford, MA) and 1 mmol/l ATP at 37 °C for 15 min. Reaction was stopped by adding LDS sample buffer and then boiling at 70 °C for 10 min. The supernatant was used for immunoblotting and the level of Akt phosphorylation at Ser473 was measured as an index of mTORC2 activity or IKKe activity.

**Induction and detection of cell death:** Experiments were performed at 48h after siRNA transfection in NRVM. NRVM was serum-starved for 18 h before the start of experiments. To induce cell death, NRVM were incubated with 100 µmol/L H2O2 for 16 h. One hour before the addition of H2O2, cells were pretreated with insulin, or vehicle. At the end of experiments, supernatant was collected and LDH activity was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

**Statistics:** All data are presented as means ± SEM. Statistical significance (P<0.05) was determined between groups using ANOVA for multiple groups or Student t test for 2 groups.
### Supplementary Table. Hemodynamic parameters

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Data are mean±SEM. * P<0.05 DMSO Control. † P<0.05 vs. DMSO IPC
HR= heart rate, LVDP= left ventricular developed pressure, CF= coronary flow,
Baseline= 20 min after stabilization, Reperfusion= 120 min after reperfusion

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K, Miura T. Hypertensive hypertrophied myocardium is vulnerable to infarction and
Online Figure I. Changes in Akt, GSKβ, eNOS, and Rps6 phosphorylation in response to inhibition of the PI3K/mTOR pathway in perfused mouse hearts at baseline.

Treatment protocol for tissue sampling (A), representative immunoblots (B) and summarized data (C) are shown. The following drugs were infused to perfused mouse hearts: Ku63794 1 µmol/L, pp242 0.5 µmol/L, Rapamycin 1 nmol/L, Wortmannin 200 nmol/L. Levels of phosphorylated proteins were normalized to α-tubulin levels. N = 4 in each group. *P<0.05 vs. control.
Online Figure II. Effect of a mTOR inhibitor on IPC-induced p70S6K phosphorylation.
Representative immunoblots and summarized data are shown. IPC was induced as shown in Figure 1A. Ku63794 (1 µmol/L) were infused from 5 min before the IPC protocol until the end of the IPC protocol. Levels of phosphorylated proteins were normalized to total p70S6K levels. Black bar = Control, Gray bar = IPC. N = 4 in each group. *P<0.05 vs. control. †P<0.05 vs. IPC no inhibitor.
Online Figure III. Effect of a mTOR inhibitor on IPC-induced IKKe activation

Representative immunoblots and summarized data are shown. IPC was induced as shown in Figure 1A. Ku63794 (1 µmol/L) was infused from 5 min before the IPC protocol until the end of the IPC protocol. Immunoprecipitated IKKe from heart homogenates was incubated with recombinant Akt and phosphorylation of recombinant Akt was examined as an index of IKKe activity. Black bar = Control, Gray bar = IPC. N = 4 in each group. *P<0.05 vs. control.
Online Figure IV. Comparison between immunoblots stained with p-RXRXX(S*/T*) antibody and p-Rps6 antibody.

Representative immunoblots are shown. A, Intensities of several bands, detected by p-Akt substrate antibody, were increased by IPC. The bands for the 32kDa p-RXRXX(S*/T*) and for p-Rps6 migrated in exactly the same location on the gel and the intensity changed in parallel. B, We transfected HA-tagged Rps6\(^{S235A/S236A}\), which was replaced by Ala at 235 and 236 on RpS6, and its control (Rps6\(^{WT}\)) in HEK293 cells. Rps6\(^{WT}\) but not Rps6\(^{S235A/S236A}\) was clearly detected by immunoblotting with p-RXRXX(S*/T*) antibody.
Online Figure V. Effects of diazoxide, 8Br-cAMP, and 8-CPT-cAMP on Rps6 phosphorylation in serum-depleted neonatal rat ventricular myocytes (NRVMs).

Representative immunoblots and summarized data for effect of diazoxide (A), 8Br-cAMP (B), and 8-CPT-cAMP (C) on phosphorylation of Rps6 at Ser235/236 and GSK3β at Ser9. A, Chelerythrine (5 µmol/L) was added to the culture medium 30 min before the treatment with diazoxide or DMSO. Samples were taken after the incubation with diazoxide or DMSO for 30 min. N = 4 in each group. *P<0.05 vs. control. †P<0.05 vs. Diazoxide 100 µmol/L. B and C, 8Br-cAMP, a PKA activator (B), or 8-CPT-cAMP, a PKG activator (C), were applied to the culture medium for 30 min. N = 3 in each group. *P<0.05 vs. control.
Online Figure VI. The time-course of a 32kDa RXRXX(S*/T*) phosphorylation during ischemia/reperfusion.

A, Ischemia/reperfusion protocol for tissue sampling. I = Ischemia, R = Reperfusion. B and C, Representative immunoblots for p-RXRXX(S*/T*) antibody (B) and p-GSK3β (C) are presented. α-tubulin and GSK3β were used as a loading control. D, The time course of IPC-induced 32 kDa RXRXX(S*/T*) phosphorylation. Black bar = Control, Gray bar = IPC. * P<0.05 vs. Control. N = 4 in each group.
**Online Figure VII. Effect of active Akt on Rps6 phosphorylation in vitro**

Representative immunoblots are shown. Rps6 was immunoprecipitated from HEK293 cells transfected with HA-Rps6$^{WT}$. Immunoprecipitated Rps6 was incubated with recombinant active or unactive Akt and phosphorylation of Rps6 was examined.
Online Figure VIII. The effect of insulin on the interaction of ribosomal proteins with mTORC2.

Representative blots (A) and summarized data (B) are shown. HEK293 cells were stimulated with insulin (200 nmol/L) after 16 h of serum starvation. Representative immunoblots and summarized data are presented. IP = Immunoprecipitation. N = 3 in each group.
Online Figure IX. The effect of a mTOR inhibitor on insulin-induced signaling in NRVM.

Representative blots are shown. NRVM was stimulated with insulin (200 nmol/L) after 16 h of serum starvation. Half an hour before the addition of insulin or vehicle, cells were incubated with pp242 or DMSO at indicated doses.
Online Figure X. Effect of Rps6 overexpression on mTORC2 signaling

A and B, Representative immunoblots (A) and summarized data (B) showing the effect of Rps6 overexpression on Akt signaling in HEK293 cells. Samples were taken at 48hr after the transfection with HA-Rps6 WT or control. Levels of phosphorylated kinases were normalized to total kinase levels. N = 4 in each group. *P<0.05 vs. Control.
Online Figure XI.  **siRNA-mediated knockdown of rictor in NRVM**

Representative immunoblots are shown. NRVM was transfected with control or rictor siRNA. Sampling for immunoblotting was performed 48hr after transfection.
Online Figure XII. Effects of Rps6 knockdown on expression of components of mTORC2, protein kinases, a protein phosphatase, and a ribosomal protein. 

A and B, The effect of siRNA-mediated knockdown of Rps6 in HEK293 cells (A) and NRVM (B). N = 5 in each group. *P<0.05 vs. control siRNA-transfected cells. C, Lysates from NRVM were analyzed by immunoblotting using indicated antibodies. Representative immunoblots are shown.
Online Figure XIII. p53 expression in NRVM.

A, The effect of Rps6 knockdown on p53 expression level in NRVM. Samples were taken at 40 h after transfection. Lysate from HEK293 cells served as positive control. Although deficiency of ribosomal proteins including Rps6 can induce p53-dependent cell cycle arrest and apoptosis (Panić L, et al. Mol Cell Biol. 2006;26:8880-8891.), p53 was not detected in NRVM transfected with control siRNA and Rps6 siRNA under our conditions. B, The effect of adriamycin on p53 expression in NRVM. p53 became detectable after adriamycin treatment. Representative immunoblots are shown. Adriamycin was applied to the culture medium for 24 h. ADR = Adriamycin.
Online Figure XIV. Schematic presentation of effects of rapamycin and Ku63794, a mTOR inhibitor, on insulin- and IPC-induced cytoprotective signaling.

A. The effect of IPC and insulin on PI3K/Akt signaling. B and C. The effects of rapamycin (B) and Ku63794 (C) on insulin- and IPC-induced cytoprotective signaling. Outlined molecules with a white background or a red background are down-regulated or up-regulated, respectively, in response to inhibitors. The two pools of mTORC2 are indicated by (1) and (2); (1) is the Rps6/ribosome-independent mTORC2 pool and (2) is the ribosomal mTORC2. Rapamycin reduces mTORC1 activity and then decreases phosphorylation of p70S6K and Rps6. As a result, rapamycin reduces Rps6-dependent ribosomal mTORC2 activity (2) through reduction of Rps6. On the other hand, the activity of PI3K is enhanced by mTORC1 inhibition due to rapamycin treatment through disruption of negative feedback mechanism on PI3K/Akt signaling (indicated with red line). Activation of mTORC2 by IPC and insulin is maintained by further activation of the PIP3-dependent, Rps6/ribosome-independent mTORC2 pathway (1) despite reduction of ribosomal mTORC2 activity by rapamycin (2). For these reasons, the infarct size limiting effect of IPC is not blocked by rapamycin. In contrast, mTOR inhibitors such as Ku63794 block mTORC2 activity in both pools, (1) and (2), leading to loss of the cardioprotective effect by IPC.
Online Figure XV. Summary of proposed roles of Rps6 and mTORC2 in cardioprotection.