SUPPLEMENTAL MATERIAL

The pivotal role of mTORC2 and involvement of ribosomal protein S6

in cardioprotective signaling

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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, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.75 and glucose 10 mmol/L). The buffer was gassed with 95% O_2/ 5% CO_2, 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/mm^2 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% CO_2 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: AAAGUUUAGCAUUCUGCag) 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^{WT} plasmid and the HA-Rps6$^{S235A/S236A}$ 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_2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 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_3VO_4, 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

mmol/L magnesium chloride. The beads with immunoprecipitated mTORC2 or IKKε 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 them 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 IKKε 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 H_2O_2 for 16 h. One hour before the addition of H_2O_2, 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

	n	Baseline	Reperfusion
HR (bpm)			
DMSO Control	7	369±13	317± 9
DMSO IPC	7	362±14	308± 8
Wortmannin Control	5	351±16	280±16
Wortmannin IPC	7	376±17	277±11
Ku63794 Control	5	352±20	331±30
Ku63794 IPC	6	369±19	306±21
pp242 Control	5	377±37	349±40
pp242 IPC	6	345±18	307±17
Rapamycin Control	5	352±19	308± 7
Rapamycin IPC	6	359±26	313±15
LVDP (cmH_2O)			
DMSO Control	7	110± 4	55± 8
DMSO IPC	7	108± 8	94± 8*
Wortmannin Control	5	113± 3	45± 8
Wortmannin IPC	7	111± 8	65± 8†
Ku63794 Control	5	117± 4	43± 8
Ku63794 IPC	6	116± 7	77± 4
pp242 Control	5	114±10	52±12
pp242 IPC	6	109± 5	69± 7
Rapamycin Control	5	107± 4	48± 4
Rapamycin IPC	6	113± 3	87± 3*
CF (ml/min)			
DMSO Control	7	2.5±0.4	1.5±0.2
DMSO IPC	7	2.7±0.4	1.9±0.3
Wortmannin Control	5	2.3±0.2	1.4±0.2
Wortmannin IPC	7	2.7±0.3	1.6±0.3
Ku63794 Control	5	2.5±0.5	1.5±0.2
Ku63794 IPC	6	2.5±0.2	1.5±0.3
pp242 Control	5	2.4±0.3	1.6±0.4
pp242 IPC	6	2.6±0.2	1.6±0.2
Rapamycin Control	5	2.1±0.3	1.6±0.1
Rapamycin IPC	6	2.4±0.3	1.8±0.3

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

References

(1) Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. Circ Res. 2007;101:1155-1163.

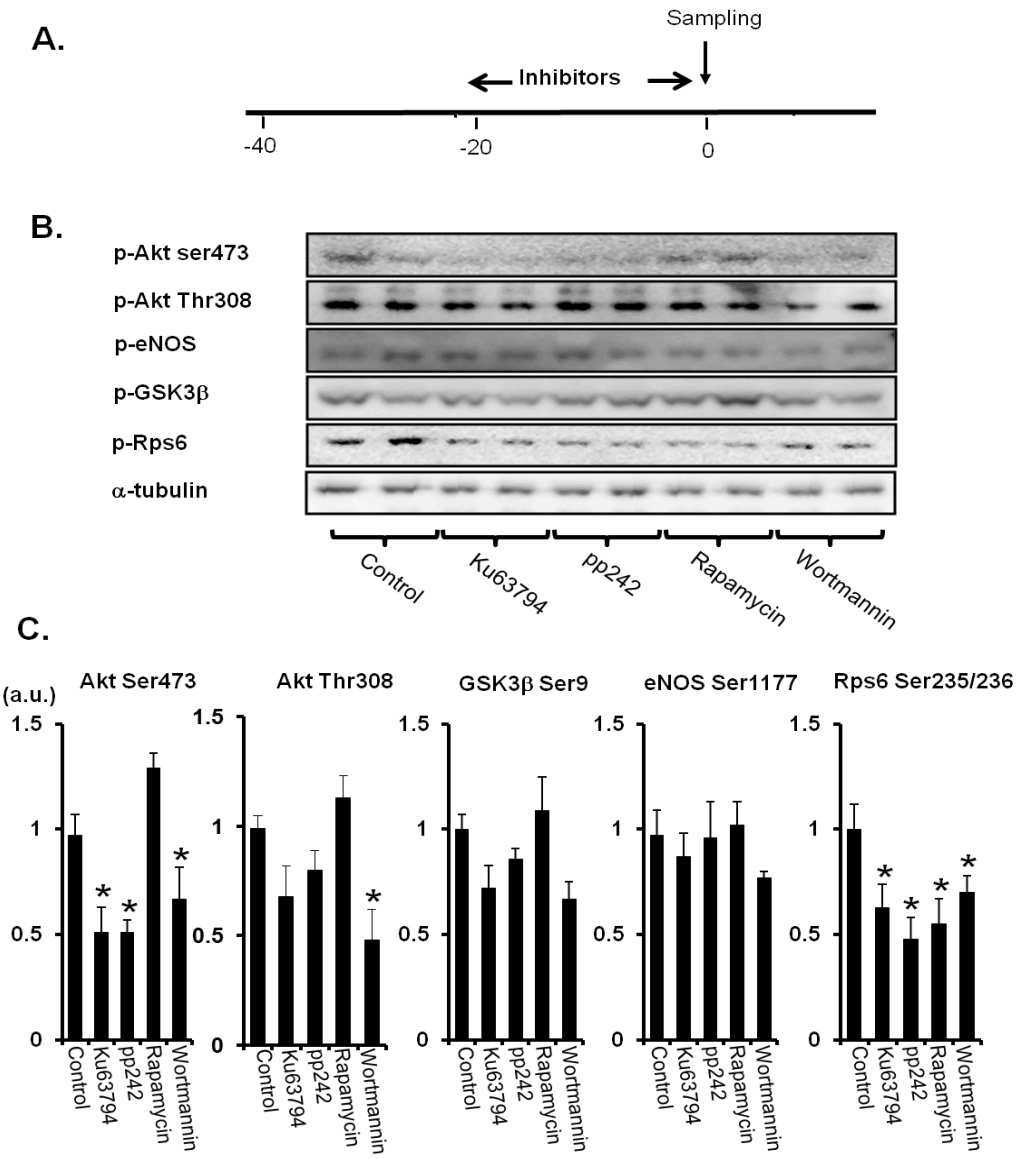
(2) Yano T, Miura T, Whittaker P, Miki T, Sakamoto J, Nakamura Y, Ichikawa Y, Ikeda Y, Kobayashi H, Ohori K, Shimamoto K. Macrophage colony-stimulating factor treatment after myocardial infarction attenuates left ventricular dysfunction by accelerating infarct repair. J Am Coll Cardiol. 2006;47:626-634.

(3) Ferlito M, Fulton WB, Zauher MA, Marbán E, Steenbergen C, Lowenstein CJ. VAMP-1, VAMP-2, and syntaxin-4 regulate ANP release from cardiac myocytes. J Mol Cell Cardiol. 2010;49:791-800.

(4) Mok KW, Mruk DD, Silvestrini B, Cheng CY. rpS6 Regulates blood-testis barrier dynamics by affecting F-actin organization and protein recruitment. Endocrinology. 2012;153:5036-5048.

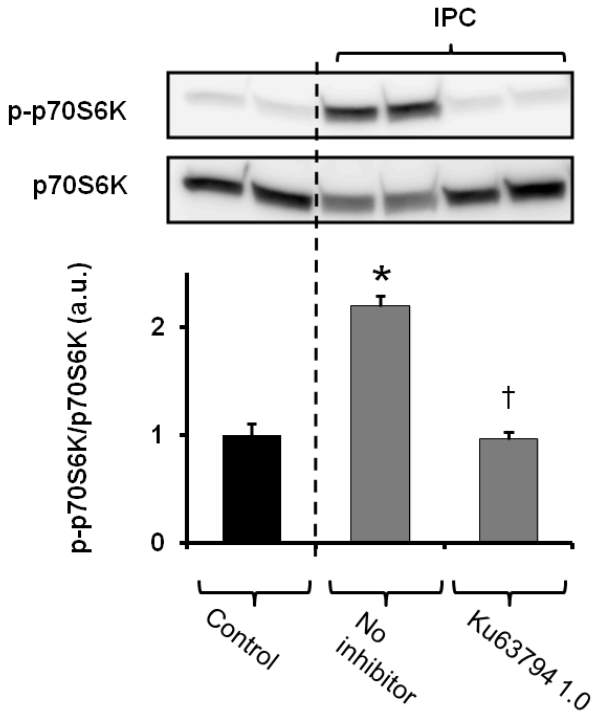
(5) Sun J, Kohr MJ, Nguyen T, Aponte AM, Connelly PS, Esfahani SG, Gucek M, Daniels MP, Steenbergen C, Murphy E. Disruption of caveolae blocks ischemic preconditioning-mediated S-nitrosylation of mitochondrial proteins. Antioxid Redox Signal. 2012;16:45-56.

(6) Yano T, Miki T, Tanno M, Kuno A, Itoh T, Takada A, Sato T, Kouzu H, Shimamoto K, Miura T. Hypertensive hypertrophied myocardium is vulnerable to infarction and refractory to erythropoietin-induced protection. Hypertension. 2011;57:110-115.

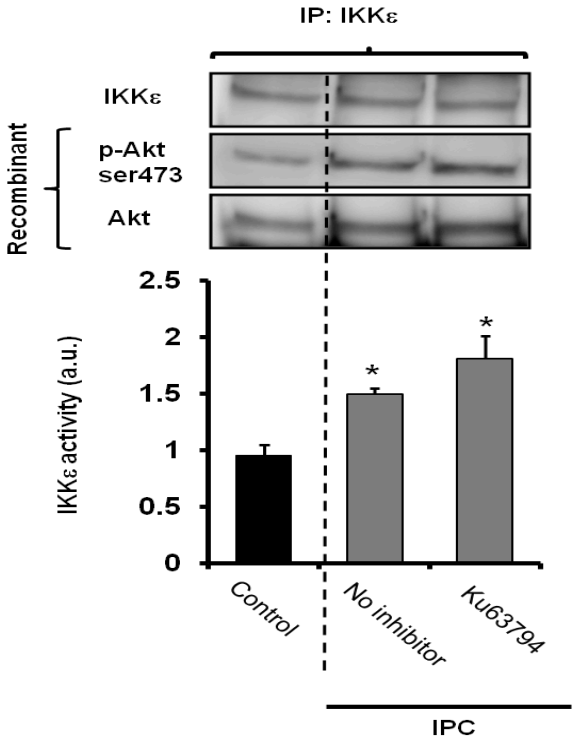


Online Figure I. Changes in Akt, GSK3β, eNOS, and Rps6 phosphorylation in response to inhibition of the PI3K/mTOR pathway in perfused mouse hearts at baseline.

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.

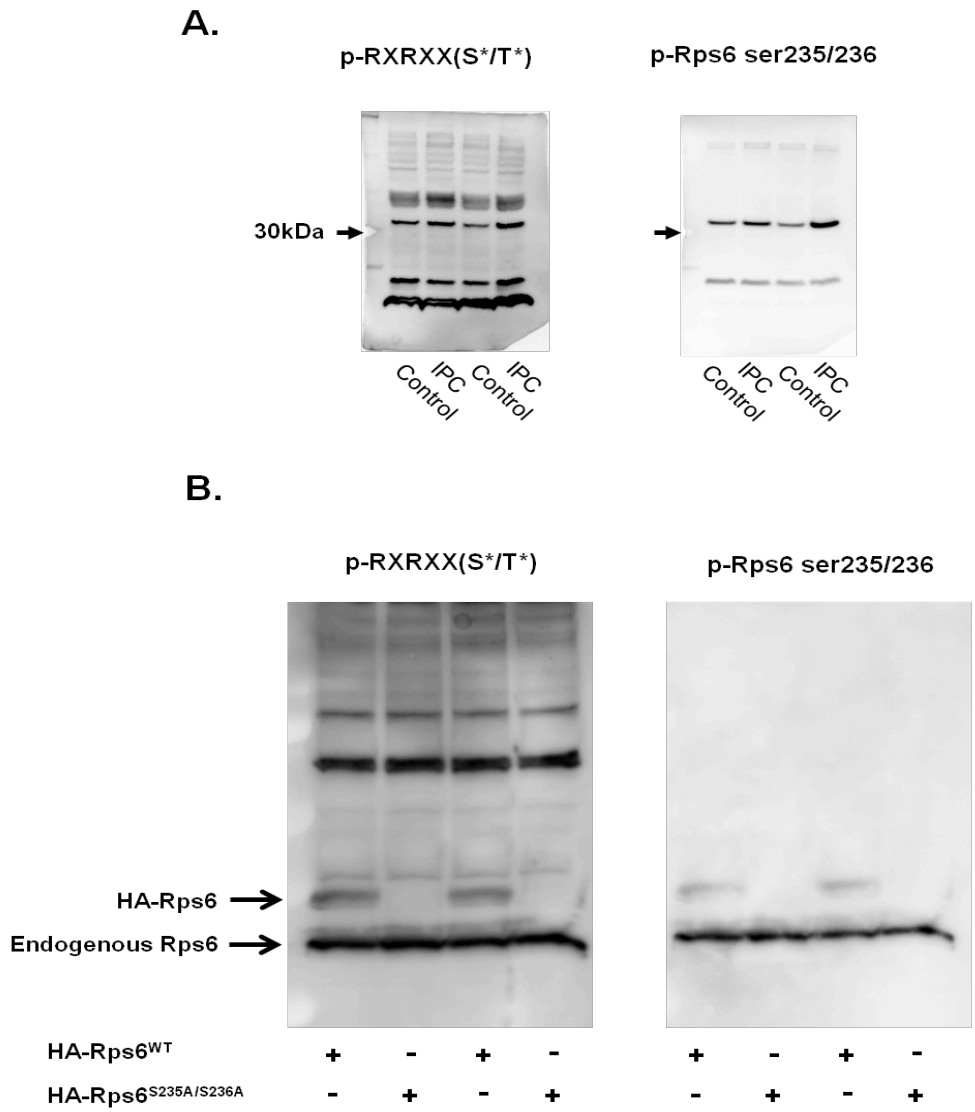


Online Figure II. Effect of a mTOR inhibitor on IPC-induced p70S6K phosphorylation. Representative immunoblots and summarized data are shown. IPC was induced as shown in Figure 1A. Ku63794 (1 μmol/L) were infused from 5 min before the IPC protocol until the end of the IPC protocol. Levels of phosphorylated 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.



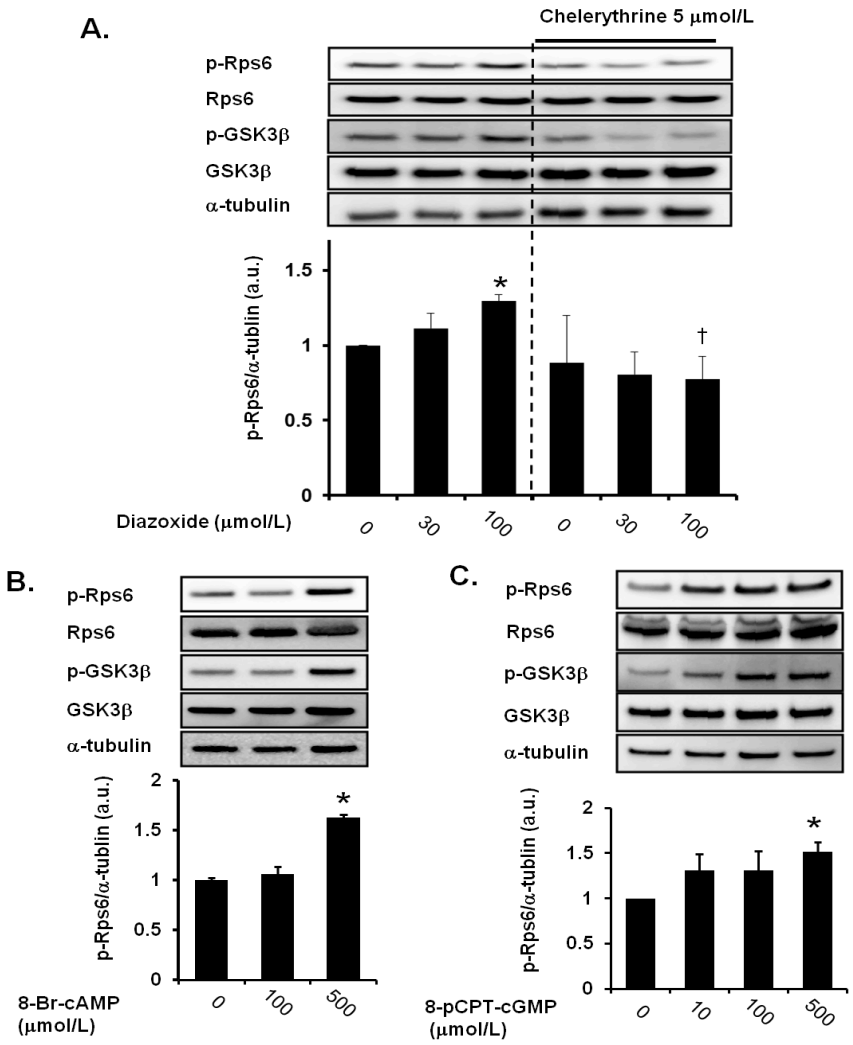
Online Figure III. Effect of a mTOR inhibitor on IPC-induced IKKε activation

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 IKKε from heart homogenates was incubated with recombinant Akt and phosphorylation of recombinant Akt was examined as an index of IKKε activity. Black bar = Control, Gray bar = IPC. $N = 4$ in each group. *$P<0.05$ vs. control.



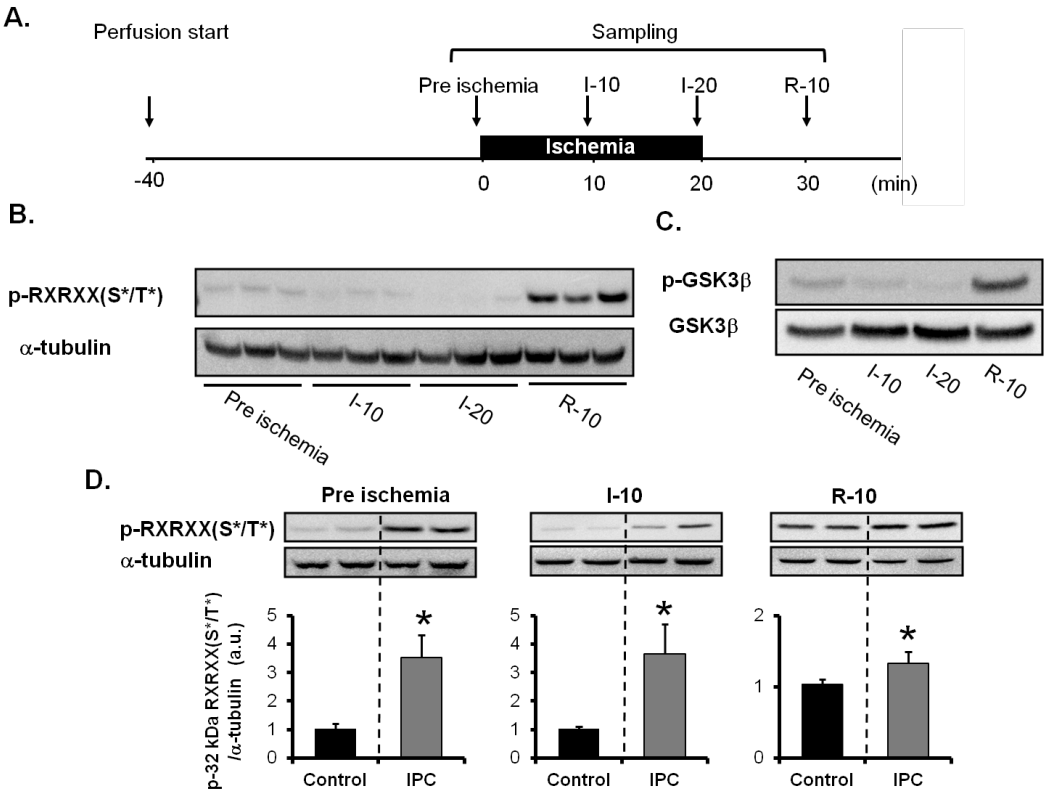
Online Figure IV. Comparison between immunoblots stained with p-RXRXX(S*/T*) antibody and p-Rps6 antibody.

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.



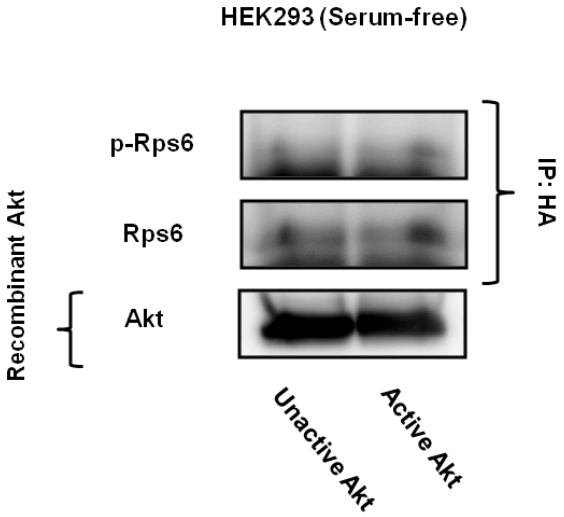
Online Figure V. Effects of diazoxide, 8Br-cAMP, and 8-CPT-cAMP on Rps6 phosphorylation in serum-depleted neonatal rat ventricular myocytes (NRVMs).

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.



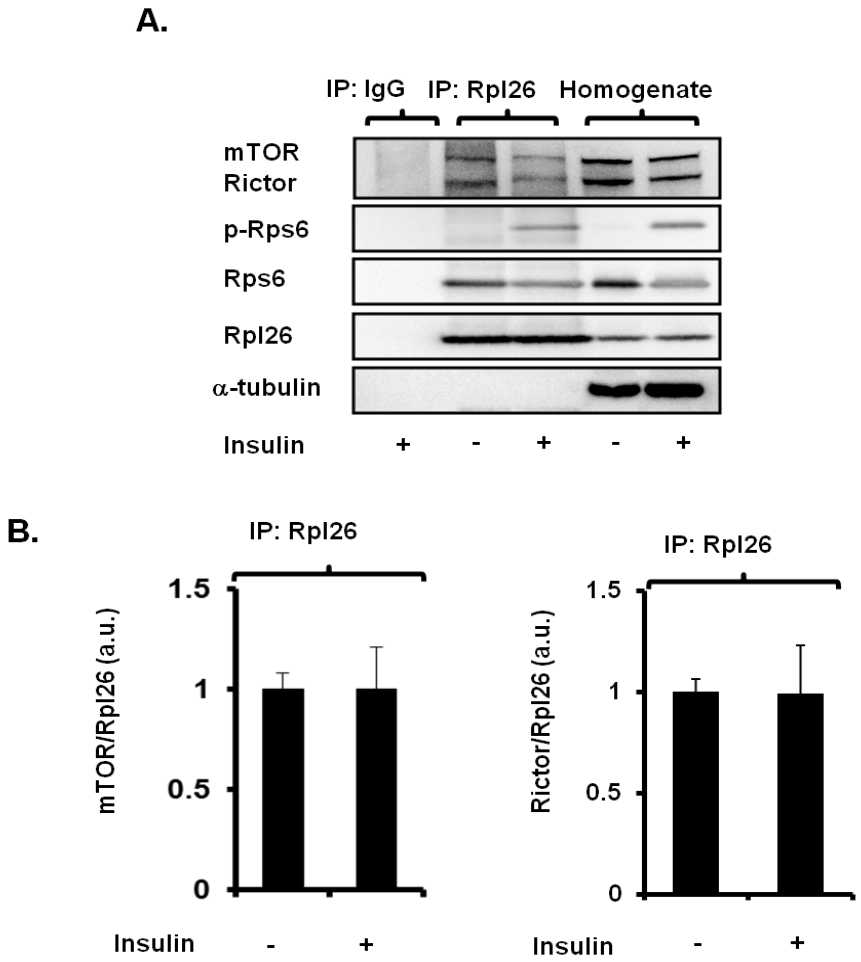
Online Figure VI. The time-course of a 32kDa RXRXX(S*/T*) phosphorylation during ischemia/reperfusion.

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.



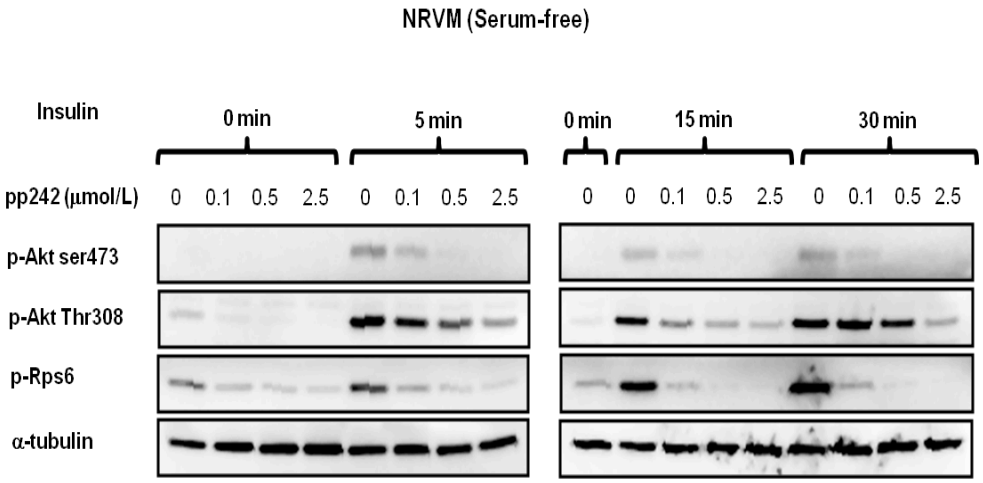
Online Figure VII. Effect of active Akt on Rps6 phosphorylation in vitro

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.



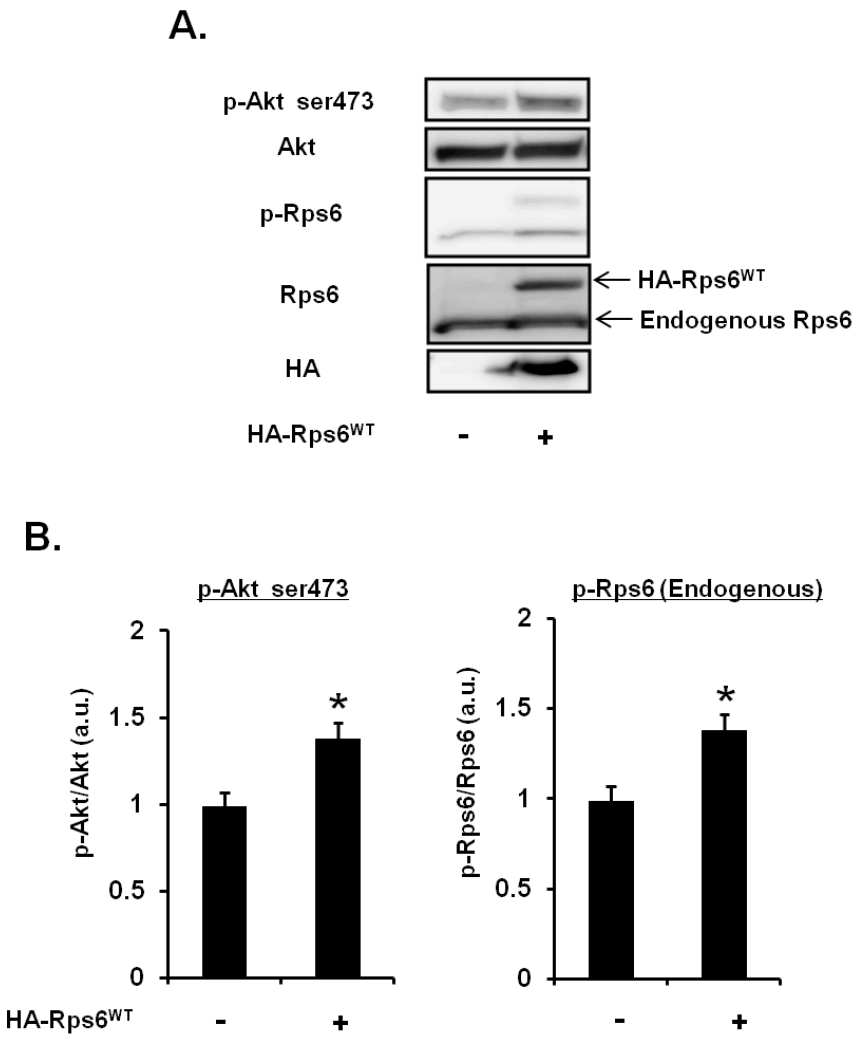
Online Figure VIII. The effect of insulin on the interaction of ribosomal proteins with mTORC2.

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.



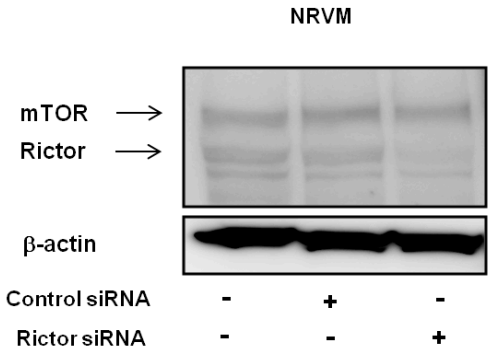
Online Figure IX. The effect of a mTOR inhibitor on insulin-induced signaling in NRVM.

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.



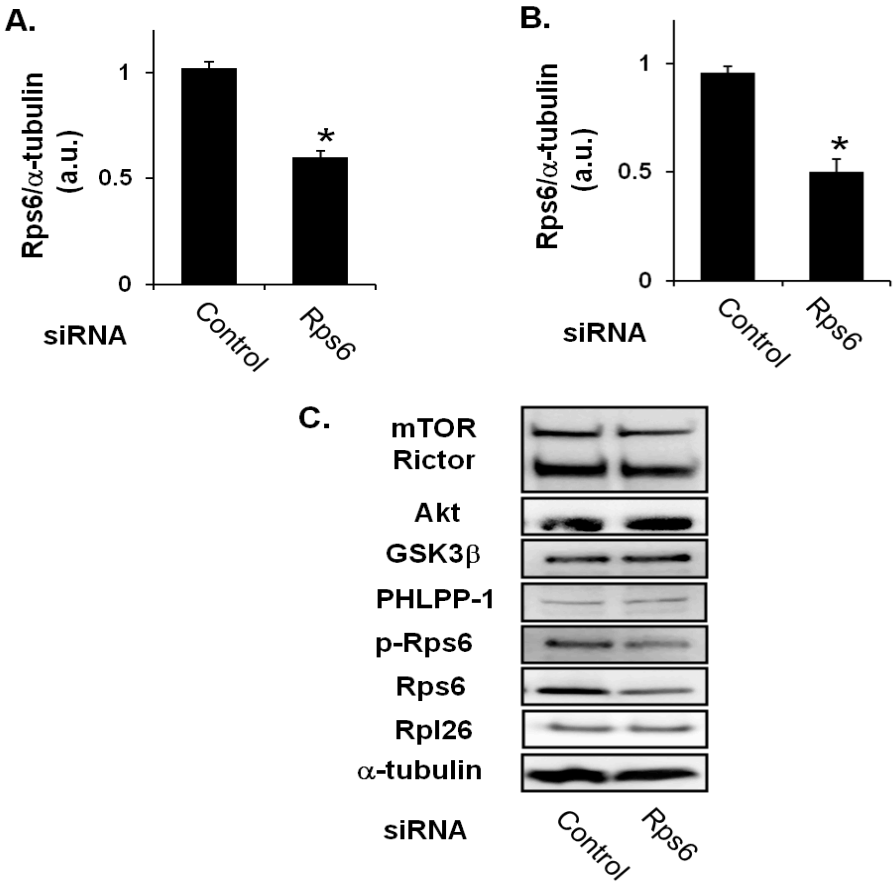
Online Figure X. Effect of Rps6 overexpression on mTORC2 signaling

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.



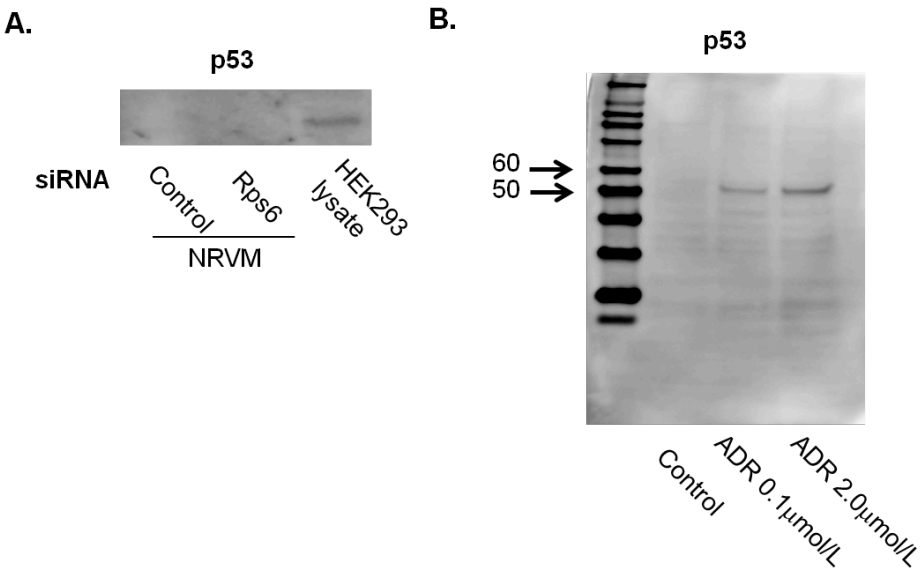
Online Figure XI. siRNA-mediated knockdown of rictor in NRVM

Representative immunoblots are shown. NRVM was transfected with control or rictor siRNA. Sampling for immunoblotting was performed 48hr after transfection.



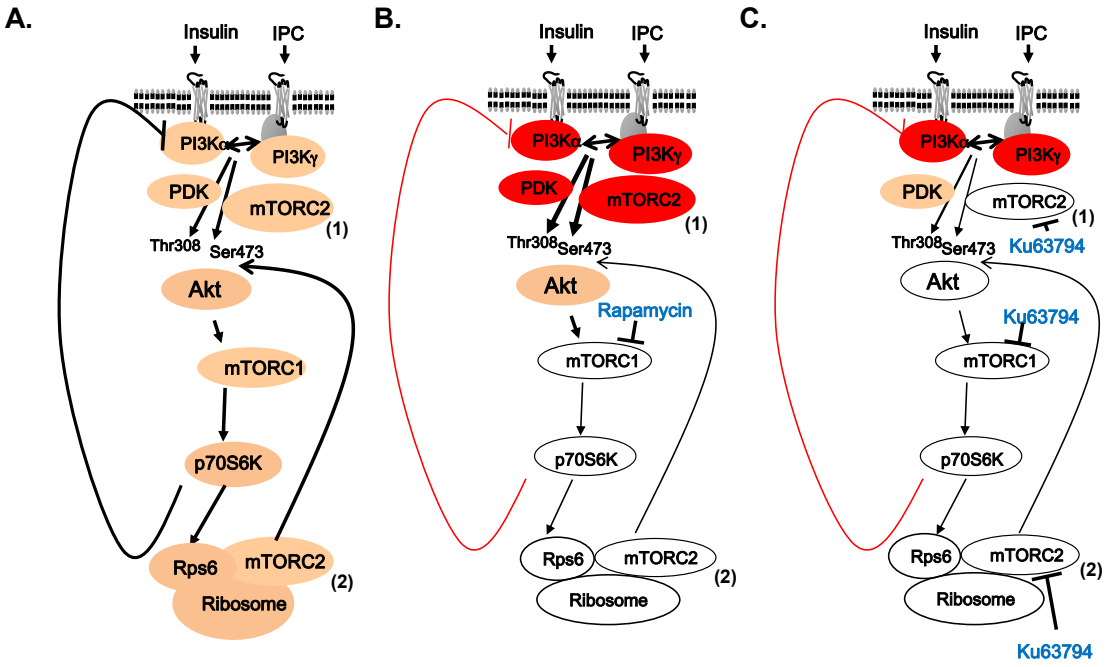
Online Figure XII. Effects of Rps6 knockdown on expression of components of mTORC2, protein kinases, a protein phosphatase, and a ribosomal protein.

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.



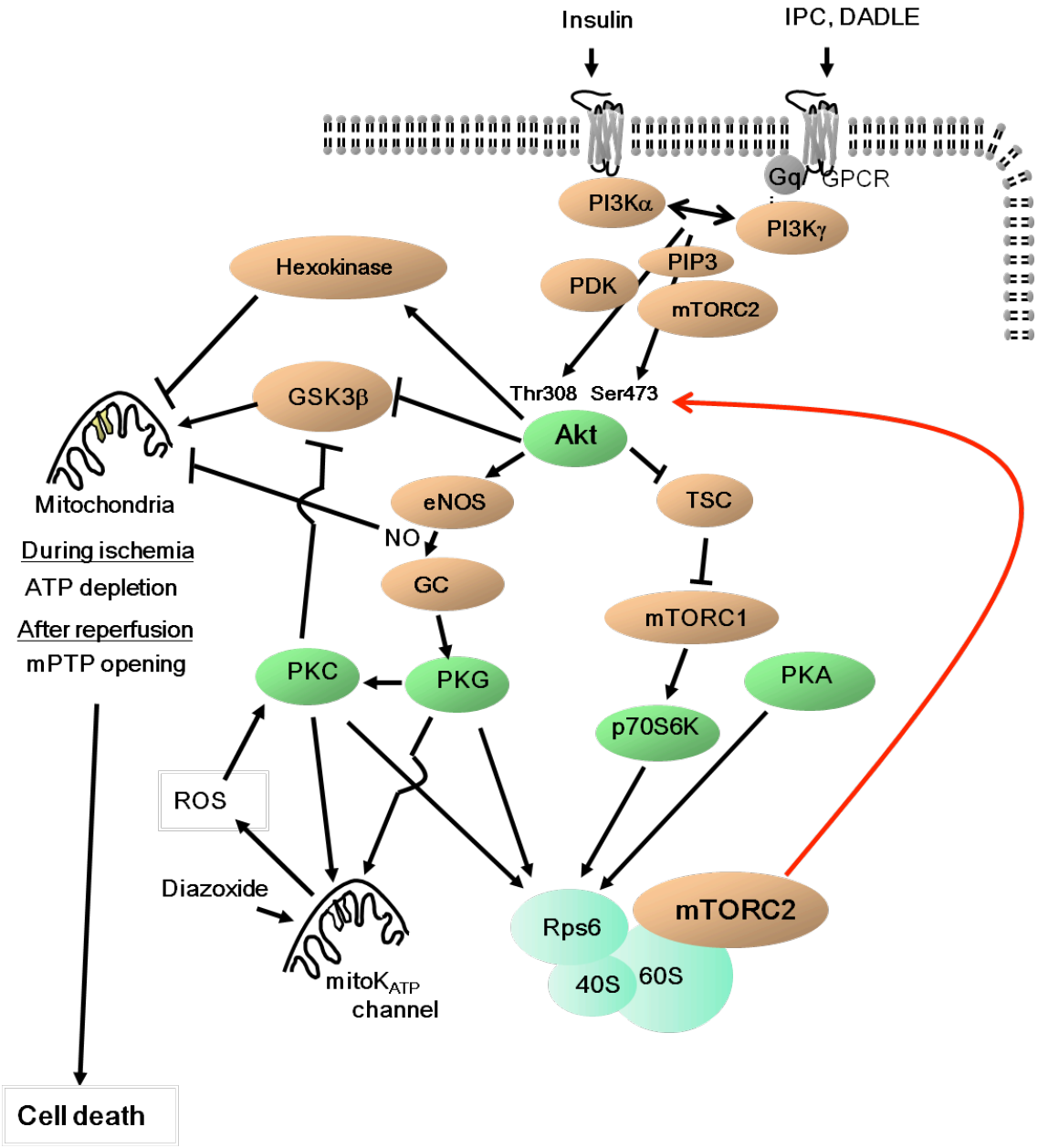
Online Figure XIII. p53 expression in NRVM.

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.