Mammalian Target of Rapamycin Cell Signaling Pathway Contributes to the Protective Effects of Ischemic Postconditioning Against Stroke

Rong Xie, MD, PhD*; Peng Wang, PhD*; Michelle Cheng, PhD; Robert Sapolsky, PhD; Xunming Ji, MD, PhD; Heng Zhao, PhD

Background and Purpose—Whether the mammalian target of rapamycin (mTOR) pathway is protective against brain injury from stroke or is detrimental is controversial, and whether it is involved in the protective effects of ischemic postconditioning (IPC) against stroke is unreported. Our study focuses on the protective role of mTOR against neuronal injury after stroke with and without IPC.

Methods—We used both an in vitro oxygen–glucose deprivation model with a mixed neuronal culture and hypoxic postconditioning, as well as an in vivo stroke model with IPC. Rapamycin, a specific pharmacological inhibitor of mTOR, and mTOR short hairpin RNA lentiviral vectors were used to inhibit mTOR activity. A lentiviral vector expressing S6K1, a downstream molecule of mTOR, was used to confirm the protective effects of mTOR. Infarct sizes were measured and protein levels were examined by Western blot.

Results—We report that stroke resulted in reduced levels of phosphorylated proteins in the mTOR pathway, including S6K1, S6, and 4EBP1, and that IPC increased these proteins. mTOR inhibition, both by the mTOR inhibitor rapamycin and by mTOR short hairpin RNA, worsened ischemic outcomes in vitro and in vivo and abolished the protective effects of hypoxic postconditioning and IPC on neuronal death in vitro and brain injury size in vivo. Overexpression of S6K1 mediated by lentiviral vectors significantly attenuated brain infarction.

Conclusions—mTOR plays a crucial protective role in brain damage after stroke and contributes to the protective effects of IPC. **(*Stroke*. 2014;45:2769-2776.)**

Key Words: ischemic postconditioning ■ stroke

The protective effects of ischemic postconditioning (IPC)—the interruption of reperfusion after stroke—are well established in various stroke models,[1] yet the underlying protective mechanisms are poorly understood.[2] The mammalian target of rapamycin (mTOR) signaling pathway plays a central role in metabolism, cell growth, differentiation, development, and cell survival.[3] A few studies have shown that brain injury induced by ischemia involves the mTOR pathway.[4–9] Whether it is beneficial or detrimental to neuronal survival remains controversial,[10] and its contribution to the neuroprotective effects of IPC has not been reported.

mTOR has a large molecular weight of ≈ 289 kDa and belongs to the phosphatidylinositol 3-kinase–related kinase family[11] (Figure 1A). Many triggers modulate mTOR activity, which is assessed by phosphorylation levels of mTOR. mTOR activity is inhibited by hypoxia, adenosine triphosphate depletion, and DNA damage, but activated by nutrients (amino acids) and growth factors. These inhibitory or stimulatory factors also modulate Akt, which is known to protect against ischemic brain injury,[10] probably through activation of the mTOR cell signaling pathway. mTOR forms 2 complexes, mTOR complex 1 and mTOR complex 2, to regulate mTOR activity.[11] mTOR complex 1 function has been extensively studied, whereas mTOR complex 2 function remains elusive. mTOR complex 1 components include PRAS40, mTOR, and Raptor. The 2 most studied mTOR substrates are S6K1 and 4EBP1. S6K1 results in S6 phosphorylation, which leads to protein synthesis.[12] mTOR phosphorylates 4EBP1, which is then released from eIF-4E, allowing the latter to promote protein synthesis and cell survival.[3,11,13]

As we and others have shown, IPC improves glucose uptake, reduces free radical generation,[14] inhibits inflammation,[15,16]

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From the Departments of Neurosurgery (R.X., P.W., M.C., R.S., H.Z.) and Biological Sciences (M.C., R.S.), and Stanford Stroke Center (H.Z.), Stanford University, CA; Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China (R.X.); and Department of Neurosurgery, Xuanwu Hospital, Capital Medical School, Beijing, China (P.W., X.J.).

*Drs Xie and Wang contributed equally.

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Correspondence to Heng Zhao, PhD, Department of Neurosurgery, Stanford University School of Medicine, 1201 Welch Rd, Room 306, MSLS Bldg, Stanford, CA 94305-5327, E-mail hzhao@stanford.edu or Xunming Ji, MD, PhD, Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, China, E-mail jixm70@hotmail.com

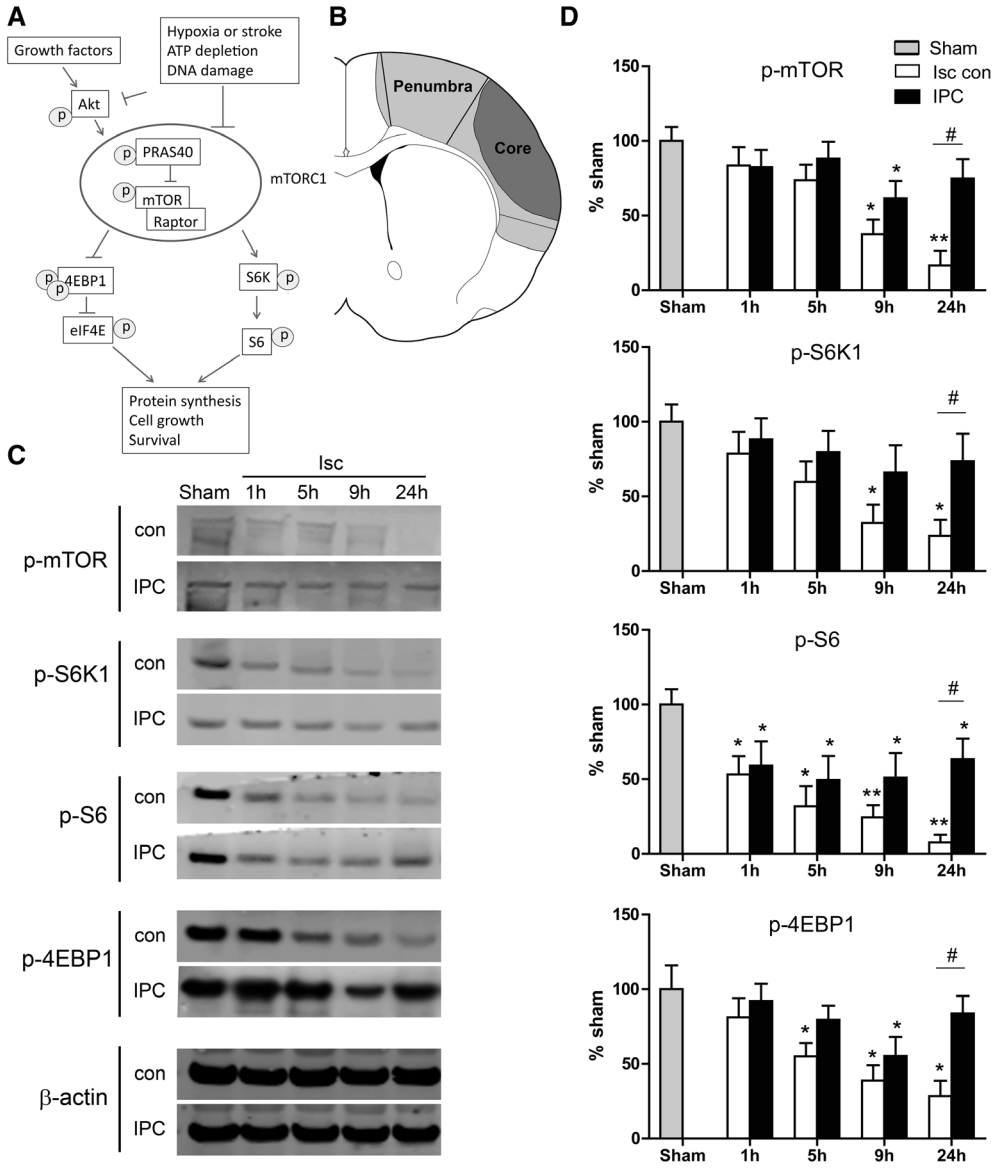

Figure 1. Effects of ischemic postconditioning (IPC) on protein expression in the mammalian target of rapamycin (mTOR) pathway. **A**, Diagram showing the major proteins in the mTOR cell signaling pathway. P indicates phosphorylation. **B**, Brain schematic showing the ischemic penumbra and core regions. The ischemic penumbra, which represents tissue spared by IPC, was dissected for Western blot analysis. The core is the area of infarct that received IPC. **C**, Representative protein bands of a Western blot for p-mTOR, p-S6K1, p-S6, and p-4EBP1 at 1, 5, 9, and 24 h poststroke. β-Actin indicates equal protein loading. **D**, Relative optical densities of protein bands, normalized to those in the sham group and expressed as percentages. *, **vs sham; $P<0.05$, 0.01, respectively; #$P<0.05$, between the 2 indicated groups; n=8 per group.

and promotes protein activity in the phosphatidylinositol 3-kinase/Akt pathway,[17] and, as introduced above, these factors all modulate mTOR activity. We hypothesized that IPC protects the brain by modulating mTOR activity. In this study, we examined whether mTOR plays a crucial role in the protective effect of IPC against stroke.

Methods

Methods are detailed in the online-only Data Supplement. Animal experiments were conducted according to protocols approved by the Stanford Institutional Animal Care and Use Committee and National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Construction of Lentiviral Vectors

As we were unable to clone the mTOR gene into the plasmid backbone, we instead constructed lentiviral vectors expressing S6K1 and enhanced green fluorescent protein; S6K1 is a downstream protein of mTOR and an indicator of mTOR activity. The control vector was a lentiviral plasmid backbone with only enhanced green fluorescent protein inserted without the S6K1 sequence. In addition, we used lentiviral vectors containing mTOR short hairpin RNA (shRNA) to inhibit mTOR expression and a scrambled shRNA gene as a negative control for mTOR shRNA.

In Vitro Oxygen–Glucose Deprivation Model and Studies

Primary mixed neuronal cultures were prepared from rat fetal brains, and experiments were performed on days 9 to 11 after preparation.

Oxygen–glucose deprivation (OGD) was induced for 6 hours in a hypoxic chamber, and in vitro hypoxic postconditioning (HPC) was performed by 3 cycles of 15-minute restoration of glucose and oxygen and 15-minute OGD. Cell viability was quantified by measuring lactate dehydrogenase release 18 hours after OGD restoration using a previously described colorimetric assay.[18] The effects of rapamycin, mTOR shRNA, and S6K1 vectors on neuronal injury were studied.

Focal Cerebral Ischemia and IPC Model

Focal cerebral ischemia was generated by 30-minute occlusion of the bilateral common carotid arteries with permanent occlusion of the distal middle cerebral artery in male Sprague–Dawley rats (300–350 g) as described previously.[14] Immediately after bilateral common carotid artery release, IPC was performed by 30 seconds of common carotid artery suture release followed by 10 seconds of occlusion, which was repeated 3×, as described.[14]

For in vivo drug and lentiviral vector injections, the drug and viral vectors were coded to blind the surgeon performing both the virus injections and stroke models. Rapamycin (5 μL, 1 mmol/L) was infused into the ventricular space ipsilateral to the ischemia using a microsyringe pump controller 1 hour before ischemia. Lentiviruses were injected by a 10-μL needle into the left cortex 5 days before ischemia. Infarct sizes were assessed 2 days after stroke using cresyl violet staining. The area of infarcted cortex was measured by a person who was blinded to the animal's condition, normalized to the contralateral cortex, and expressed as a percentage, as described previously.[14]

Protein Preparation and Western Blot

To confirm the efficacy of gene transfer of S6K1 and knockdown effects of mTOR shRNA, the mixed neuron cells were grown in 6-well plates and transfected with lentiviral vectors. The cells were harvested 48 hours after gene transfer, and proteins were extracted for Western blotting.

To study the effects of rapamycin and IPC on protein levels in the mTOR pathway, animals were euthanized at 1, 5, 9, and 24 hours after stroke (n=8). A sham surgery group was used as the control. Brain tissues from the ischemic penumbra were harvested to detect the expression of proteins in the mTOR pathway by Western blot (Figure 1). However, to study the effects of gene transfer on protein expression, brain tissue 1 mm in diameter around the needle track was dissected for Western blotting.

Statistical Analysis

Graphpad Prism 5.0 software was used for statistical analyses. One-way or 2-way ANOVA was used followed by the Fisher least significant difference post hoc test. Tests were considered significant at P values <0.05. Data are presented as mean±SEM.

Results

IPC Attenuated Stroke-Induced Reductions in Protein Phosphorylation in the mTOR Pathway

We first characterized the effects of stroke and IPC on levels of phosphorylated mTOR, S6K1, S6, and 4EBP1 proteins in the mTOR pathway (Figure 1). In the penumbra, p-S6 levels were decreased as early as 1 hour after stroke, p-4EBP1 levels were decreased starting at 5 hours, and all 4 proteins including p-mTOR were significantly decreased 9 and 24 hours after stroke. With IPC, significant improvements were seen at 24 hours in all phosphorylated protein levels (Figure 1C and 1D). We also measured total protein levels, and they were relatively stable poststroke compared with their phosphorylated counterparts, but most were still significantly reduced at 24 hours; IPC had no significant effect on their expression (Figure I in the online-only Data Supplement).

Rapamycin Worsened Infarction In Vivo and Inhibited Protection by IPC In Vivo and by HPC In Vitro

After demonstrating that IPC promoted mTOR activity and associated phosphorylated proteins in the mTOR pathway (Figure 1), we tested whether the specific mTOR inhibitor rapamycin could block its protective effects in vitro and in vivo. In vitro, rapamycin worsened cell death induced by OGD in a mixed neuronal culture (Figure 2A). HPC mitigated this cell death, but rapamycin abolished HPC's protective effects (Figure 2A). The in vivo study confirmed that rapamycin worsens infarct sizes, with or without IPC (Figure 2B and 2C).

The effects of rapamycin on protein levels of p-mTOR, p-S6, and p-4EBP1 in animals without stroke and after stroke with or without IPC were examined (Figure 3). Rapamycin reduced these protein levels in nonischemic

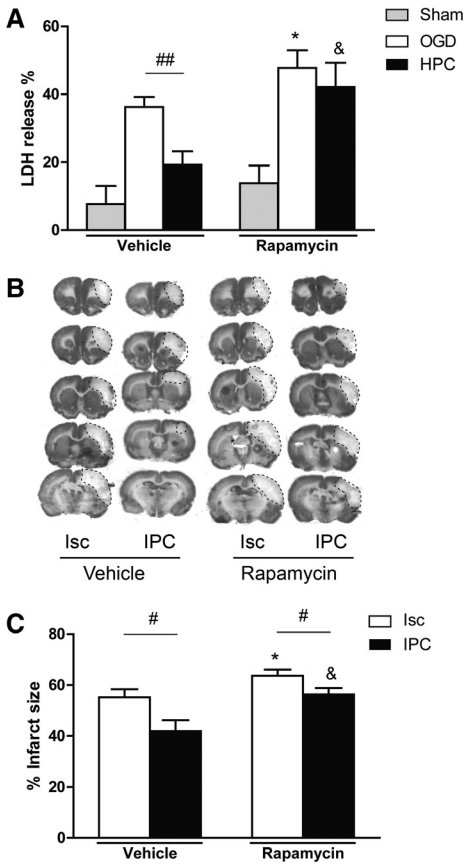

Figure 2. Rapamycin blocked the protective poststroke effects of hypoxic postconditioning (HPC) and ischemic postconditioning (IPC) both in vitro and in vivo. **A**, Effects of rapamycin and HPC on neuronal death as measured by lactate dehydrogenase (LDH) release in vitro. LDH release was measured 18 h post–oxygen–glucose deprivation (OGD). All data from cultures with OGD were normalized to the values of control, non-OGD samples treated with vehicle that did not contain rapamycin; n=16 per group. **B**, Representative coronal sections of ischemic brains stained by cresyl violet method. Infarct regions are traced. **C**, Average infarct sizes were measured from 5 coronal sections; n=8 per group. *, &vs the vehicle of the indicated group, respectively, P<0.05; #, ##P<0.05, 0.01, respectively, between the 2 indicated groups.

brains. Rapamycin also inhibited these protein levels in ischemic brains without IPC in the penumbra from 1 to 24 hours. Furthermore, rapamycin treatment significantly reduced these proteins at all time points in the ischemic penumbra with IPC (Figure 3A and 3B). Nevertheless, it did not alter total protein levels compared with vehicle (Figure II in the online-only Data Supplement).

mTOR shRNA Abolished the Protective Effects of HPC In Vitro and IPC In Vivo

Rapamycin might generate systemic effects even when injected locally if it enters the blood circulation. We used a lentiviral mTOR shRNA to further analyze the effects of mTOR inhibition on pathological and protective outcomes of stroke and IPC (Figure 4A). Western blot results indicate that mTOR shRNA transfected in cell culture successfully blocked protein expression of p-mTOR and its downstream protein p-4EBP1 (Figure 4B). Like rapamycin, transfection of mTOR shRNA worsened neuronal stroke damage both in vitro and in vivo and abolished the protective effects of HPC in vitro and IPC in vivo (Figure 4C and 4D).

We further examined the in vivo effects of mTOR shRNA on major proteins expressed in the mTOR pathway and confirmed that it reduced the protein levels of p-mTOR, p-S6K1, and p-4EBP1 in ischemic brains both with and without IPC (Figure 5). We confirmed that mTOR shRNA inhibited total protein levels of mTOR (Figure III in the online-only Data Supplement).

S6K Gene Transfer Protected Against Brain Injury

After confirming that mTOR inhibition worsened brain injury, we intended to study if gene transfer of an mTOR lentiviral vector alone would inhibit stroke-induced brain injury. When we were unable to construct an mTOR vector, likely as a result of its large molecular weight, we instead constructed a lentiviral vector of S6K1 (Figure 6A), one of the most studied proteins downstream of mTOR. We confirmed that gene transfer of the S6K1 vector resulted in the overexpression of p-S6K1 and S6K1 (Figure 6B; Figure IV in the online-only Data Supplement). Both in vitro and in vivo studies demonstrated that S6K1 gene transfer inhibited neuronal death and brain injury, but there were no synergistic effects with HPC in vitro or with IPC in vivo (Figure 6C and 6D). It is likely that IPC or S6K1 was protective to a threshold beyond which neuronal injury could not be further reduced.

Discussion

The mTOR pathway is involved in brain injury induced by ischemia; however, whether it is beneficial or detrimental

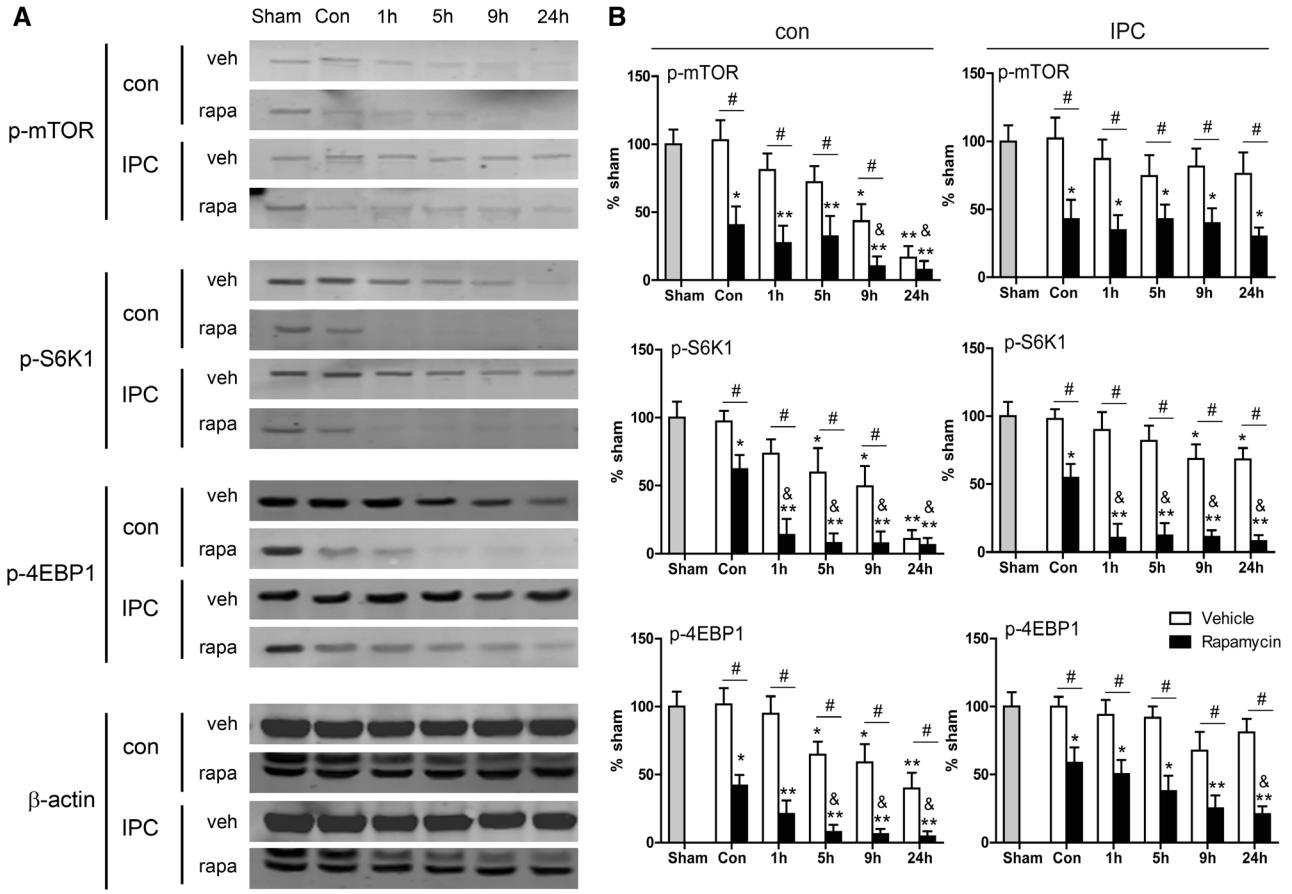

Figure 3. Effects of rapamycin on protein levels in the mammalian target of rapamycin (mTOR) pathway. **A**, Representative protein bands of major proteins in the mTOR pathway. **B**, Quantified levels of each protein. *, **vs sham brain, $P<0.05$, 0.01, respectively; &vs con (nonischemic control with rapamycin injection), $P<0.05$; #between the 2 indicated groups, $P<0.05$, 0.01, respectively; n=8 per group. IPC indicates ischemic postconditioning; and rapa, rapamycin.

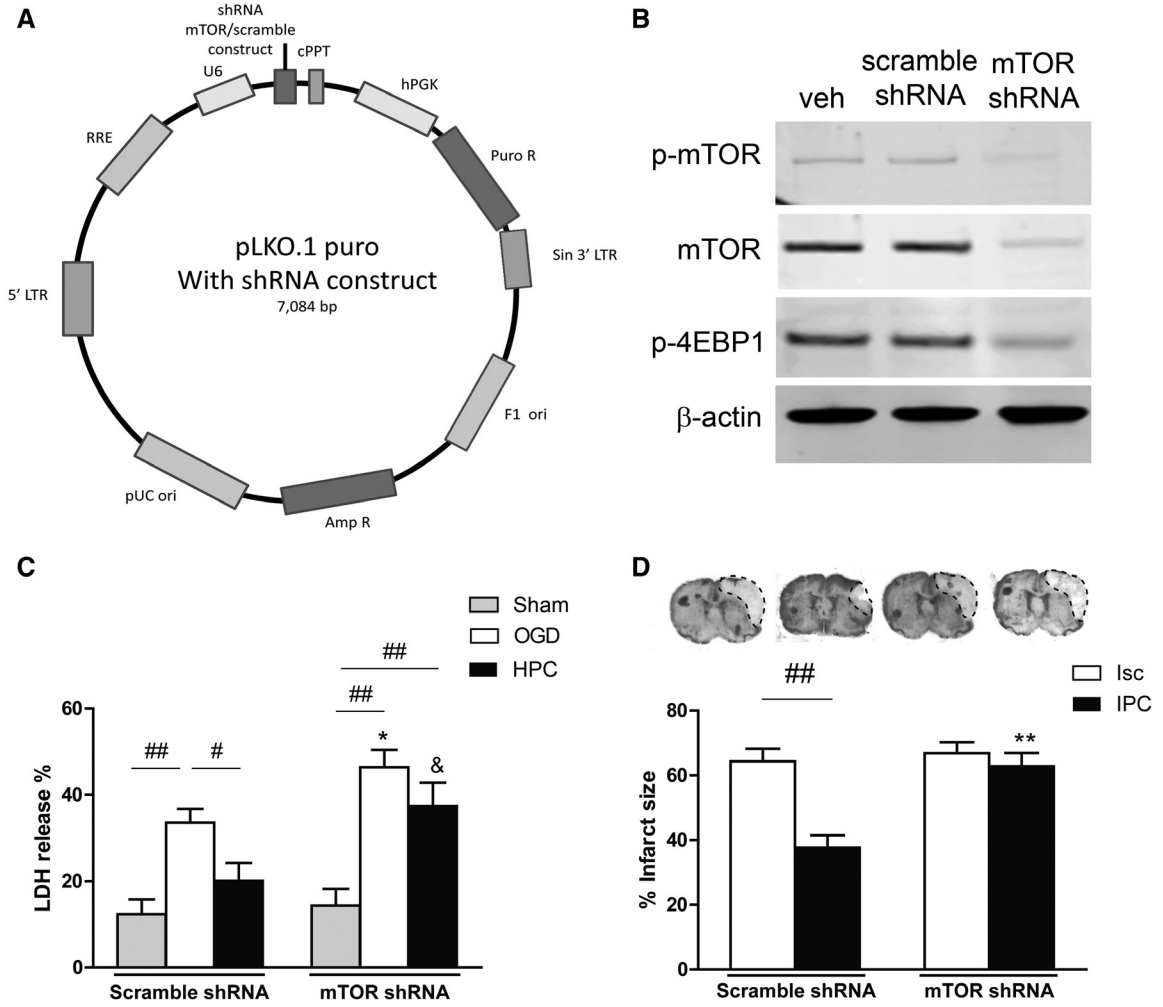

Figure 4. Detrimental effects of the mammalian target of rapamycin (mTOR) short hairpin RNA (shRNA) lentiviral vector in stroke. **A**, Schematic backbone of lentiviral vector of pLKO.1 puro with shRNA construct. **B**, Effects of gene transfer in primary neuronal cultures on protein expression of p-mTOR and p-4EBP1 in the mTOR pathway. mTOR shRNA was confirmed to reduce protein levels of p-mTOR and p-4EBP1. **C**, Effects of mTOR shRNA gene transfer on neuronal death in the oxygen–glucose deprivation (OGD) model measured by lactate dehydrogenase (LDH) release. shRNA exacerbated neuronal death in cell culture that underwent OGD and abolished the protective effect of hypoxic postconditioning (HPC). *vs OGD group with scramble shRNA, $P<0.05$; &vs post group with scramble shRNA, $P<0.05$; #, ##$P<0.05$, 0.01, between indicated 2 groups, respectively; n=16 per group. **D**, Effects of mTOR shRNA on infarct sizes in ischemic brains with or without ischemic postconditioning (IPC). **Top:** Representative coronal brain sections for infarction stained by cresyl violet. **Bottom:** Bar graphs show infarct sizes. mTOR shRNA enlarged infarction in animals treated with control stroke and abolished the protective effects of IPC. **vs scramble shRNA, ischemic group, $P<0.01$; ##$P<0.01$, between indicated 2 groups; n=8 per group.

to the ischemic brain remains controversial. There is evidence that several neuroprotectants upregulate mTOR activity, including the antioxidants melatonin and estradiol[19,20] and the anti-inflammatory agent silibinin.[7] In addition, rapamycin treatment increased cleaved caspase protein levels and promoted neuronal death after hypoxia ischemia.[21] Furthermore, S6K1 gene deletion in cultured astrocytes resulted in increased apoptosis, and S6K1 inhibition increased mortality and infarction in mice.[9] In contrast, studies have also reported that mTOR is detrimental to neuronal survival. Ghiglieri et al[22] showed that the mTOR inhibitor rapamycin prevented the induction of postischemic long-term potentiation in an in vitro OGD model in brain slices and attenuated ischemic cell damage. Wang et al[6] showed that rapamycin protected against neuronal injury by autophage induction after stroke. In neonatal hypoxic ischemia, Balduini et al[23] also found that mTOR/S6K1 inhibition by rapamycin reduced neuronal injury via autophage induction.

Here we first demonstrated that the levels of multiple phosphorylated proteins in the mTOR pathway were reduced soon after stroke onset in the ischemic brain, including mTOR, S6K1, S6, and 4EBP1, suggesting that protein degradation in the mTOR pathway precedes final brain injury. Our study includes a detailed characterization of several poststroke protein levels in the mTOR pathway, whereas previous studies often investigated only 1 or 2 proteins at a single time point in the acute poststroke phase.[7,8] Shi et al[8] reported decreased levels of p-mTOR after stroke, but this was based on one time point measured at 24 hours.

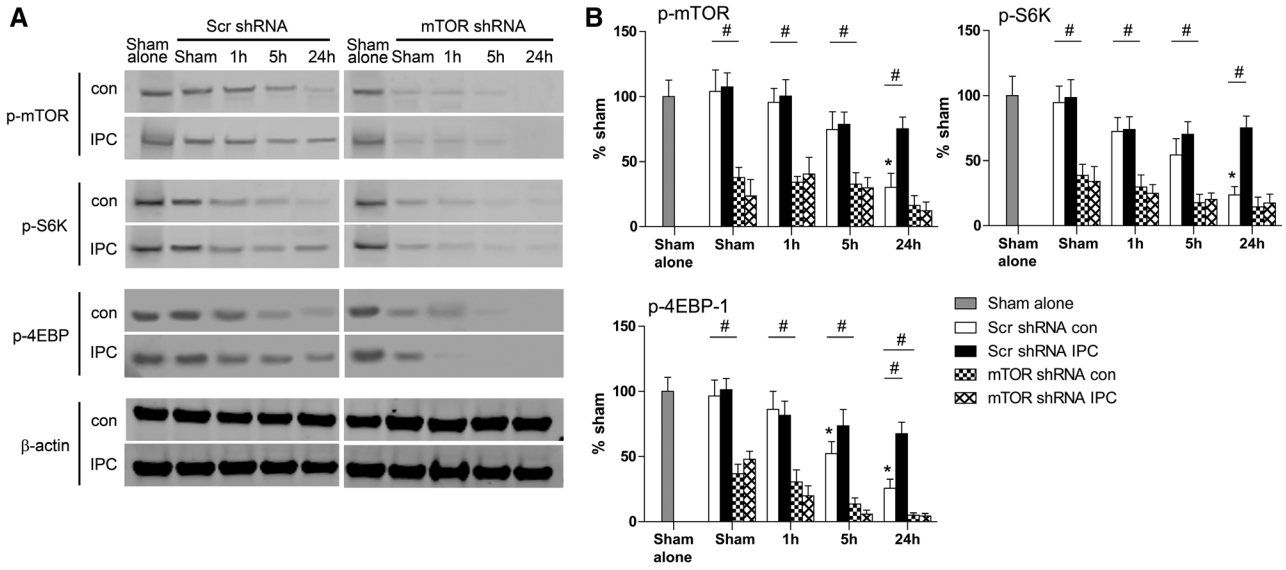

Figure 5. Effects of mammalian target of rapamycin (mTOR) short hairpin RNA on protein levels in the mTOR pathway. **A**, Representative protein bands in the mTOR pathway measured by Western blot. Brain tissues from penumbra were collected and analyzed at 1, 5, 9, and 24 h poststroke with or without ischemic postconditioning (IPC). **B**, Bar graphs show quantified levels of each protein. *vs sham brain, $P<0.05$; #$P<0.05$, between the 2 indicated groups; n=8 per group.

Pastor et al[9] showed that protein levels of p-S6K1 and p-S6 were reduced 4 to 48 hours after OGD; however, no in vivo experiments were performed. Moreover, these results conflict with other reports showing that p-S6K1 increased at day 1[7] or day 4 after stroke[4] and that p-mTOR protein expression increased at 2 to 24 hours in developing rat brains with hypoxia ischemia.[21] We systemically measured phosphorylation of major proteins in the mTOR pathway after stroke, both with and without IPC, and showed reduced phosphorylation of p-mTOR, p-S6K1, p-S6, and p-4EBP1 at multiple time points. This was attenuated by IPC, suggesting that IPC improved mTOR activity and corresponded to its protective effects. Because we and others have previously shown that Akt plays a critical role in IPC[1,17] and that Akt promotes mTOR activity,[24] we cannot exclude the possibility that IPC may improve mTOR activity via enhancement of Akt activity and that Akt may be a significant factor in the role of mTOR in IPC. Nevertheless, more experiments are required to prove this hypothesis.

We also showed for the first time that both the specific mTOR inhibitor rapamycin and mTOR shRNA enlarged infarction, and that S6K1 gene transfer reduced neuronal death in vitro and inhibited infarction in vivo, suggesting a neuroprotective role for mTOR in stroke. In most previous studies, rapamycin was the sole mTOR inhibitor used to address the effects of the mTOR pathway in stroke,[5,6,25–27] and both beneficial[5,19,20] and detrimental[6,22] effects of the mTOR pathway have been reported. In contrast, we used multiple approaches to show that both mTOR shRNA and rapamycin promoted neuronal death in vitro as well as enlarged infarction in a controlled stroke in vivo and attenuated the protective effects of IPC both in vitro and in vivo. In addition, we showed that S6K1 gene transfer reduced infarction. Taken together, our studies support a neuroprotective role for the mTOR pathway in stroke.

Although we have provided strong evidence that mTOR activity supports neuronal survival after stroke both with and without IPC, how it achieves such protection requires further investigation. IPC may reduce acute infarction by blocking apoptosis through mTOR activation, because IPC inhibits apoptosis after stroke[1] and mTOR blocks apoptosis.[28] Estradiol administration has been shown to inhibit apoptosis in the poststroke ischemic cortex by promoting the mTOR/p70S6K1 signaling pathway.[19,20] Deletion of PTEN, a tumor suppressor protein, blocked neuronal apoptosis by enhancing mTOR phosphorylation.[8] Furthermore, S6K1 gene deletion impaired Bcl-2–associated death promoter phosphorylation and decreased Bcl-2 and Bcl-xL expression in astrocytes.[10] Bcl-2–associated death promoter phosphorylation interacts with protein 14-3-3–sequestering proapoptotic proteins, and both Bcl-2 and Bcl-xL are antiapoptotic proteins that maintain mitochondrial function. Reduced p-BAD, Bcl-2, and Bcl-xL can result in mitochondria-mediated apoptosis.[10] Alternatively, the mTOR pathway may also generate neuroprotection by promoting protein synthesis, which contributes to the protective effects of IPC. We have shown that 9 to 24 hours after stroke IPC, protein levels of p-eIF4E and p-S6K1 improve, which are critical for protein synthesis.[29] Brain injury induced by stroke has been associated with protein synthesis inhibition,[30] which is regulated by mTOR, p-eIF4E, and p-S6K1 activity. IPC may enhance protein synthesis, promoting compensation for protein loss after stroke, synaptic plasticity, and injury repair.

In conclusion, we have used for the first time multiple approaches to inhibit and promote mTOR activity in the setting of stroke in vitro and in vivo and provided strong evidence that mTOR activity contributes to neuronal survival in stroke both with and without IPC or HPC. These results indicate that mTOR is a potential target for neuroprotection in stroke treatment.

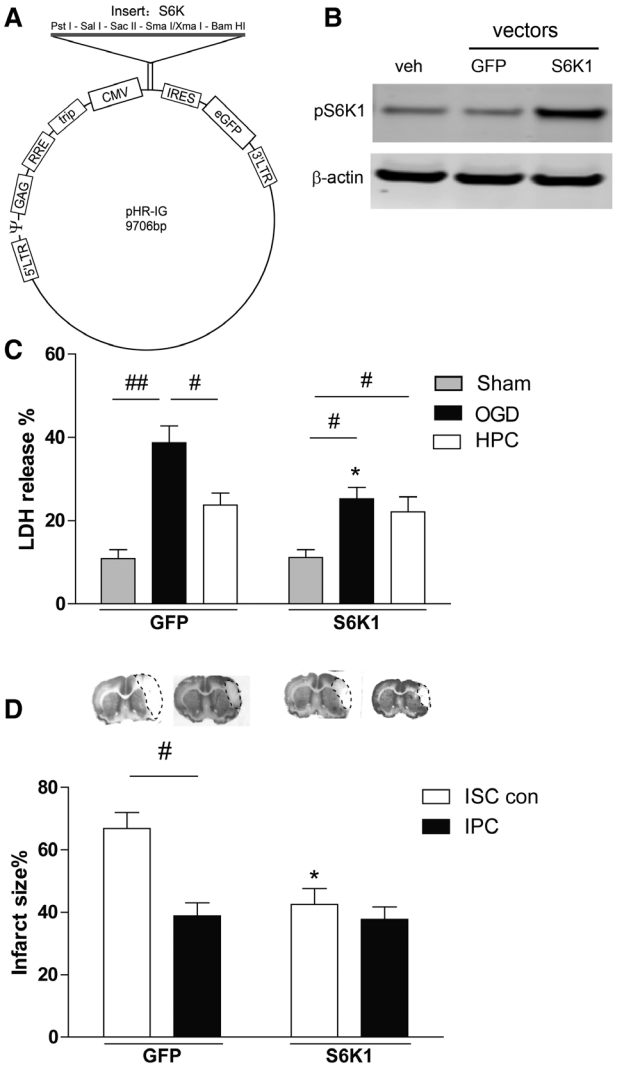

Figure 6. S6K1 lentiviral vector construction and in vitro and in vivo effects. **A**, Schematic backbone of lentiviral vector of pHR-TRIP CMV-RES-eGFP with S6K1 construction. **B**, Western blot confirmation of the effects of gene transfer in primary neuronal cultures on protein expression of p-S6K1. Gene transfer resulted in robust increases in protein expression of p-S6K1. **C**, Effects of S6K1 gene transfer on neuronal death in the oxygen–glucose deprivation (OGD) model measured by lactate dehydrogenase (LDH) release. *vs the OGD with green fluorescent protein control vectors, $P<0.05$. #, ##$P<0.05$, 0.01, respectively, between 2 indicated groups; n=16 per group. **D**, Effects of S6K1 gene transfer on infarct sizes in ischemic brains with or without ischemic postconditioning (IPC). Representative coronal sections of ischemic brains and statistical results are shown. *vs ischemic control stroke, $P<0.05$; #$P<0.05$, between 2 indicated groups; n=8 per group.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

The mTOR Cell Signaling Pathway Contributes to the Protective Effects of Ischemic Postconditioning Against Stroke

Rong Xie, MD, PhD*; Peng Wang, PhD*; Michelle Cheng, PhD; Robert Sapolsky, PhD; Xunming Ji, MD, PhD; Heng Zhao, PhD

Methods

Animal experiments were conducted according to protocols approved by the Stanford Institutional Animal Care and Use Committee (IACUC) and NIH Guidelines for Care and Use of Laboratory Animals. Animals were housed under a 12:12 hour light:dark cycle with food and water available *ad libitum*.

Construction of Lentiviral Vectors

To study the role of mTOR in brain injury induced by stroke, we first attempted to construct lentiviral vectors to overexpress mTOR, but we were unable to clone the mTOR gene into the plasmid backbone, probably because of its large molecular weight. As a substitute, we constructed lentiviral vectors expressing S6K1, which is a downstream protein of mTOR and an indicator of mTOR activity. The S6K1 gene was cloned from the plasmids of S6K1(pcDNA3) (S6K1: 26610; Addgene, Cambridge, MA) and inserted into the lentiviral backbone plasmid pHR'tripCMV-IRES-eGFP, which contains a cytomegalovirus (CMV) promoter and an IRES sequence between its multiple cloning sites and eGFP. The IRES sequence enables independent expression of both the target gene and eGFP simultaneously. NotI and XbaI restriction enzymes were used for cloning. The control vector was a lentiviral plasmid backbone with only eGFP inserted. In addition, we used lentiviral vectors containing mTOR shRNA to inhibit mTOR expression and a scramble shRNA gene as a negative control for mTOR shRNA (mTOR shRNA 1856, scramble shRNA 1864; Addgene, Cambridge, MA).

Lentiviral Vector Generation and Titration

We used a 3 plasmid system for lentivirus packaging as detailed in our previous study, which included a lentiviral transfer vector (pHR'tripCMV- IRES-eGFP) containing the coding region of the targeted genes, the packaging plasmid (p-delta) that provides all vector proteins driven by the trip CMV promoter, except the envelope protein, and the envelope-encoding plasmid that encodes the heterologous vesicular stomatitis virus envelope protein.[1] In brief, a mixture of 45 μg of transfer vectors, 30 μg of packaging plasmids, and 15 μg of envelope-encoding plasmids were transiently transfected into three T175 flasks containing 1.5 x 10^7 HEK-293T cells using the calcium phosphate precipitation method. Supernatants were collected 72 hours post-transfection and viral particles were concentrated by ultracentrifugation. Viruses were resuspended in phosphate-buffered saline (PBS) and kept at -80°C until use. The virus particles were titered with the TCID50 method as described before.[2,3] Virus titers ranged from 1x10^8 to 5 x10^8 TU/ml and were diluted in PBS to the final concentration of 1x10^8 TU/ml before gene transfer was conducted.

In Vitro OGD Model, Gene Transfer, HPC, and Cell Viability Assay

Primary mixed neuronal cultures were prepared using timed-pregnant Sprague–Dawley rats (E18; Charles River Laboratories International, Wilmington, MA). Briefly, rats were anesthetized with isoflurane and the E18 embryos were removed. The cortical region of the fetal brains was dissected in warm media and pooled together. The cortices were titrated and incubated in papain solution (10 U/ml, Worthington Biochemical Corporation, Lakewood, NJ) for 20 minutes at

37°C, then centrifuged at 1500 rpm for 5 minutes at room temperature. Cells were resuspended in minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal horse serum (Hyclone, Logan, UT), 2 mmol/L glutamine (Gibco), 25 mM glucose, and 1% penicillin/streptomycin (Gibco). Cells were plated onto poly-D-lysine-coated tissue culture plates at 7.5 x 10^5 cells/ml. Medium was completely changed after 24 hours. One-half medium changes were performed at day 4. Cultures were incubated at 37°C in a 5% CO_2 incubator and experiments were performed on days 9 to 11.

To study the effects of rapamycin on neuronal death, rapamycin (100 nM) was added to cell cultures and the same amount of PBS (vehicle) was added as a control 2 days before OGD induction. A sham (control) group without OGD was also included.

To study the effects of S6K1 and mTOR shRNA vectors on neuronal injury, the lentiviral vectors, diluted with PBS to 5 µl for 24-well plates and to 10 µl for 6-well plates, were added directly to the medium of 9-day primary mixed neuron cultures with the multiplicity of infection at 5. The same amount of PBS was also added to the medium as an experimental control when testing the efficiencies of S6K1 gene expression and mTOR shRNA on protein expression.

Cells were then incubated at 37°C in a 5% CO_2 incubator for another 2 days before undergoing OGD to induce in vitro ischemia. Primary mixed neuron cultures were washed twice with glucose-free balanced salt solution (BSS0, pH 7.4) and the plates were transferred to a modular hypoxic chamber filled with mixed gases of 5% CO_2 and 95% N_2. The cells were kept in the hypoxic chamber for 6 hours at 37°C with an oxygen level < 0.02%. Cultures were then restored with glucose to a final concentration of 5.5 mM (BSS5.5, pH 7.4) and recovered under

normoxic conditions (37°C, 5% CO_2) for 18 hours (OGD restoration). The control groups without OGD were washed twice with 5.5 mM glucose in BSS5.5 (pH 7.4).

In vitro HPC was performed as previously described.[4] After 6 hours of OGD, before the cultures were fully restored with glucose (BSS5.5, pH 7.4) and recovered under normoxic oxygen conditions (37°C, 5% CO_2), they underwent 3 cycles of 15-minute restoration (restored with BSS5.5 and normoxic oxygen conditions) and 15-minute OGD, followed by 18 hours of restoration under regular growth conditions.

Cell viability was quantified by measuring LDH release 18 hours after OGD restoration using a previously described colorimetric assay.[5] Briefly, 100 μl of cell-free supernatant was transferred to 96-well plates. The supernatant was incubated with 150 μl of nicotinamide adenine dinucleotide/phosphate buffer (0.15 mg/mL) for 10 minutes. Then 30 μl of sodium pyruvate (2.97 mg/mL) was added and the absorbance wavelength was measured at 340 nm using a microplate reader. Background absorbance was subtracted and the percentage of LDH release was calculated based on an LDH standard curve.

Focal Cerebral Ischemia and IPC Model

Focal cerebral ischemia was generated as described previously.[6-9] Anesthesia in male Sprague-Dawley rats (300-350 g) was induced by 5% isoflurane and maintained by 1% to 2% isoflurane throughout the experiment. Core body temperatures were monitored with a rectal probe and maintained at 37°C. The distal middle cerebral artery (MCA) was exposed and permanently cauterized above the rhinal fissure. The bilateral CCAs were transiently occluded

for 30 minutes while the distal MCA remained permanently occluded. Immediately after bilateral CCA release, IPC was performed by 30 seconds of CCA suture release followed by 10 seconds of occlusion, which was repeated 3 times as previously described.[6,7,9]

Rapamycin Injection In Vivo

Rapamycin (Calbiochem, Billerica, MA), dissolved in PBS to a final concentration of 1 mM, was used to study the effects of mTOR inhibition on infarction, protein expression, and behavioral tests in animals subjected to stroke with or without IPC. One hour prior to ischemia, animals were anesthetized and placed in stereotactic frames. Either 5 µl of drug solution or vehicle (PBS) was infused into the ventricular space ipsilateral to the ischemia using a microsyringe pump controller at 0.5 µl/minute (World Precision Instruments, Inc., Sarasota, FL) (from bregma: anteroposterior, 0.92 mm; mediolateral, 1.5 mm; dorsoventral, 3.5 mm).

In Vivo Gene Transfer

For in vivo lentiviral vector injections, all viral vectors were coded to blind the surgeon to the stroke models. Lentiviruses were injected into the left cortex 5 days prior to ischemia. While under isoflurane anesthesia rats were placed in a stereotaxic frame. A 1-cm sagittal skin incision on the head exposed the skull. A left hemisphere burr hole was drilled according to the coordinate 0.96 mm posterior, 3.5 mm lateral to the bregma. A 10-µl syringe (Hamilton Company, Reno, NV) with a 26-gauge needle (Cat# 80010) was inserted into the cortex and lowered to the depth of 1.8 mm from the dura. Each viral vector (5 µl) of scramble shRNA,

mTOR shRNA, GFP or S6K1 was injected into the cortex using a microsyringe pump controller at 0.5 µl/min (World Precision Instruments). The same amount of PBS was injected as an experimental control, in addition to the scramble shRNA or GFP control vector. After viral vector administration, the needle was left for 10 minutes before being withdrawn. The wound was closed and the animals were allowed to recover.

General Histology and Infarct Size Measurement

To assess infarct sizes in rat brains receiving stroke both with and without IPC, treated with or without rapamycin, and with or without lentiviral vectors, rats at 2 days or 3 weeks after stroke were perfused transcardially with cold 0.9% saline, followed by 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were post-fixed in 4% PFA and 20% sucrose for 24 hours, cut into 5 coronal blocks, rostral (level 1) to caudal (level 5), and frozen at -80°C. For animals receiving no virus treatment, an average infarct size was calculated based on infarct sizes from all 5 levels. For animals that received a virus injection, however, the block containing the needle track in each brain was sectioned (30 µm) and mounted onto glass slides using a cryostat. The slices with a needle track were selected for infarct measurement. Successful transfection of the lentiviral vectors in the selected slices was confirmed by observing protein expression of eGFP under fluorescent confocal microscopy (LSM 510; Zeiss). Sections were stained with cresyl violet. The area of the infarcted cortex was measured by a person who was blinded to the animal's condition, normalized to the contralateral cortex, and expressed as a percentage as described previously.[9-11]

Protein Preparation for Western Blotting

To confirm the efficacy of gene transfer of S6K1 and knockdown effects of mTOR shRNA, the mixed neuron cells were grown in 6-well plates and transfected with lentiviral vectors. The cells were harvested 48 hours after gene transfer and homogenized in the cold cell extraction buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the protease inhibitor cocktail. The homogenate was centrifuged at 13000 rpm for 20 minutes at 4°C and the supernatant was removed for protein detection. Protein concentrations were measured using the Bradford assay.

To study the effects of IPC on protein levels in the mTOR pathway, animals subjected to stroke with or without IPC were sacrificed at 1, 5, 9, and 24 hours after stroke (n=8). A sham surgery group was used as the control. Brain tissues in the penumbra were harvested to detect expression of the mTOR pathway by Western blot.[7,10]

To study the effects of rapamycin on protein expression with and without IPC in the acute phase after stroke in rats, rapamycin or PBS was stereotactically-injected into the right lateral ventricle of animals 1 hour before stroke and IPC. Animals were sacrificed at 1, 5, 9, and 24 hours after stroke (n=8 per group). Animals receiving sham surgery and rapamycin or PBS, but without stroke, were also prepared as control groups. Brain tissue in the penumbra and ischemic core were harvested to detect expression of the mTOR pathway by Western blot, as stated in the above paragraph. For long-term study, brain tissues in the peri-infarct region from rats receiving rapamycin treatment were collected 1 and 3 weeks after stroke.

To study the effects of gene transfer on protein expression in animals after stroke with or without IPC, brain tissues with 1 mm diameter around the needle track were dissected 1, 5, and

24 hours after stroke onset. In addition, brain tissue from animals receiving sham surgery without ischemia, with or without transfection of lentiviral vectors, was also prepared as controls for Western blotting.

Whole cell protein was extracted from the fresh brain tissue and a Western blot was performed as described, with modification.[7,10,12] Briefly, brain tissue was cut into small pieces and homogenized in a glass homogenizer using 7 volumes of the cold cell extraction buffer (FNN0001; Invitrogen, Eugene, OR), containing 1 mM PMSF and a protease inhibitor cocktail (1:20, P-2714; Sigma, St. Louis, MO). The homogenate was centrifuged at 13000rpm for 20 minutes at 4°C and the supernatant was taken for protein detection.

Western Blotting Procedures and Antibodies

For Western blot, 20 μg of protein in each lane was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4% to 15% Ready Gel (L050505A2; Bio-Rad, Hercules, CA) at 200V for 45 minutes. Protein bands were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) at 100V for 2 hours. Membranes were incubated with primary antibodies overnight at 4°C followed by Alexa Fluor 488 donkey anti-rabbit or anti-mouse immunoglobulin G secondary antibody (1:5000; Invitrogen) for 1 hour in a dark room. Manufacturers, catalog numbers, and applications of all primary antibodies are listed in the Table. Membranes were scanned using Typhoon trio (GE Healthcare, Fairfield, CT). The optical densities of all protein bands were analyzed using IMAGEQUANT 5.2 software (GE Healthcare). Samples from sham surgery, scramble shRNA, or GFP- only gene transfer were

used as the control to compare with the experimental samples.

Statistical Analysis

Graphpad Prism 5.0 software was used for statistical analyses. For infarction analyses, one-way ANOVA was used followed by the Fisher least significant difference post hoc test.

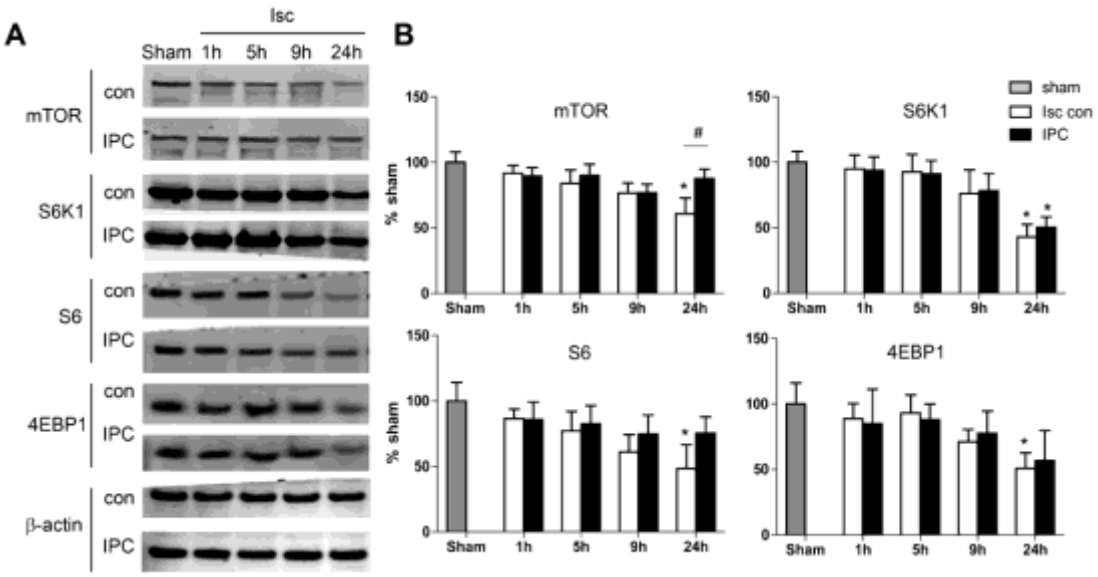
For Western blots, two-way ANOVA was used to compare the optical densities of all protein levels of penumbral area versus core at the stated time points, followed by the Fisher least significant difference post hoc test. Two-way ANOVA was also used to analyze various protein bands among postconditioning, postconditioning with rapamycin pre-treatment, and control ischemia. For behavioral tests, one-way repeated measures ANOVA was used to compare a test at different time points in the same group, and two-way ANOVA was used to compare tests among postconditioning, postconditioning with rapamycin pre-treatment, control ischemia, and sham group, followed by the Fisher least significant difference post hoc test. Tests were considered significant at P values <0.05. Data are presented as mean±SEM.

Table. *Antibodies, concentrations and manufacturers used*

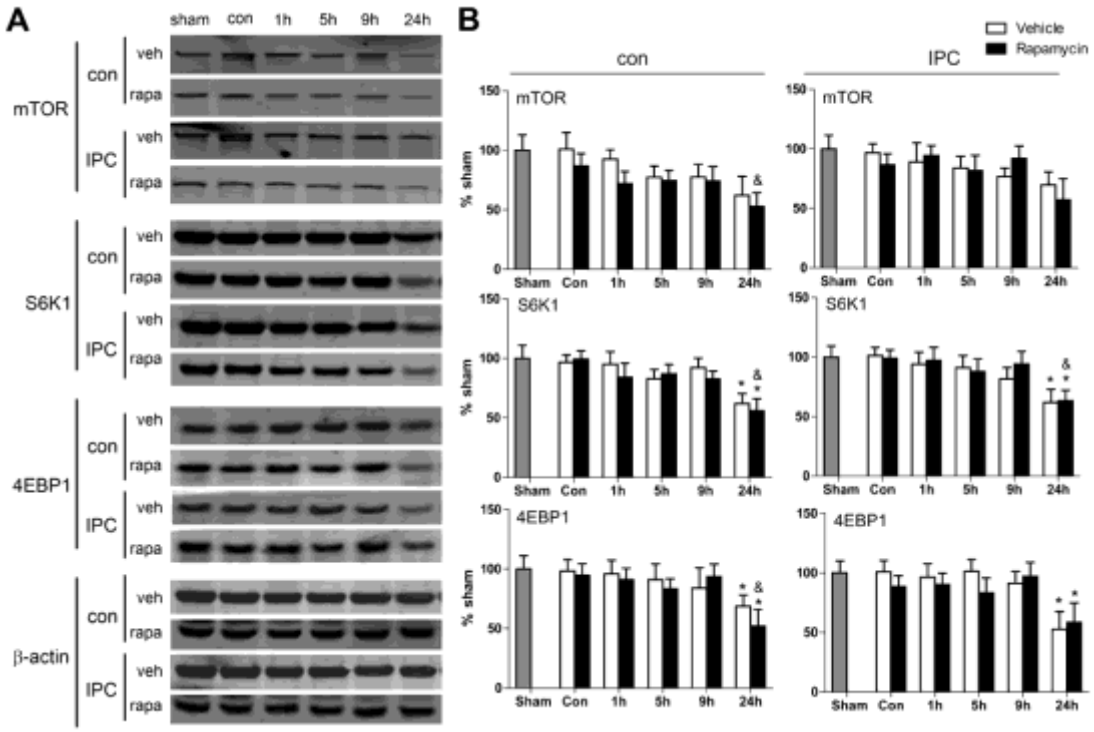
Antibodies	**Source**	**Dilutions**	**Manufacturer**	**Catalog#**	**Application**
P-Akt (Ser473)	Rabbit	1:1000	Cell Signaling	9271	WB
Akt	Rabbit	1:1000	Cell Signaling	9272	WB
Akt1	Rabbit	1:1000	Cell Signaling	2938	WB
Akt2	Rabbit	1:1000	Cell Signaling	3063	WB
Akt3	Rabbit	1:500	Cell Signaling	3788	WB
p-mTOR (Ser2448)	Rabbit	1:200/1:1000	Cell Signaling	2971	WB
mTOR	Rabbit	1:1000	Cell Signaling	2983	WB
p-S6K1 p70 (Ser371)	Rabbit	1:500	Cell Signaling	9208	WB
S6K1 p70	Rabbit	1:500	Cell Signaling	9202	WB
p-4EBP1	Rabbit	1:500	Cell Signaling	9456	WB
4-EBP1	Rabbit	1:500	Cell Signaling	9452	WB
β-actin	Mouse	1:3000	Sigma	A-5441	WB

WB = Western Blots

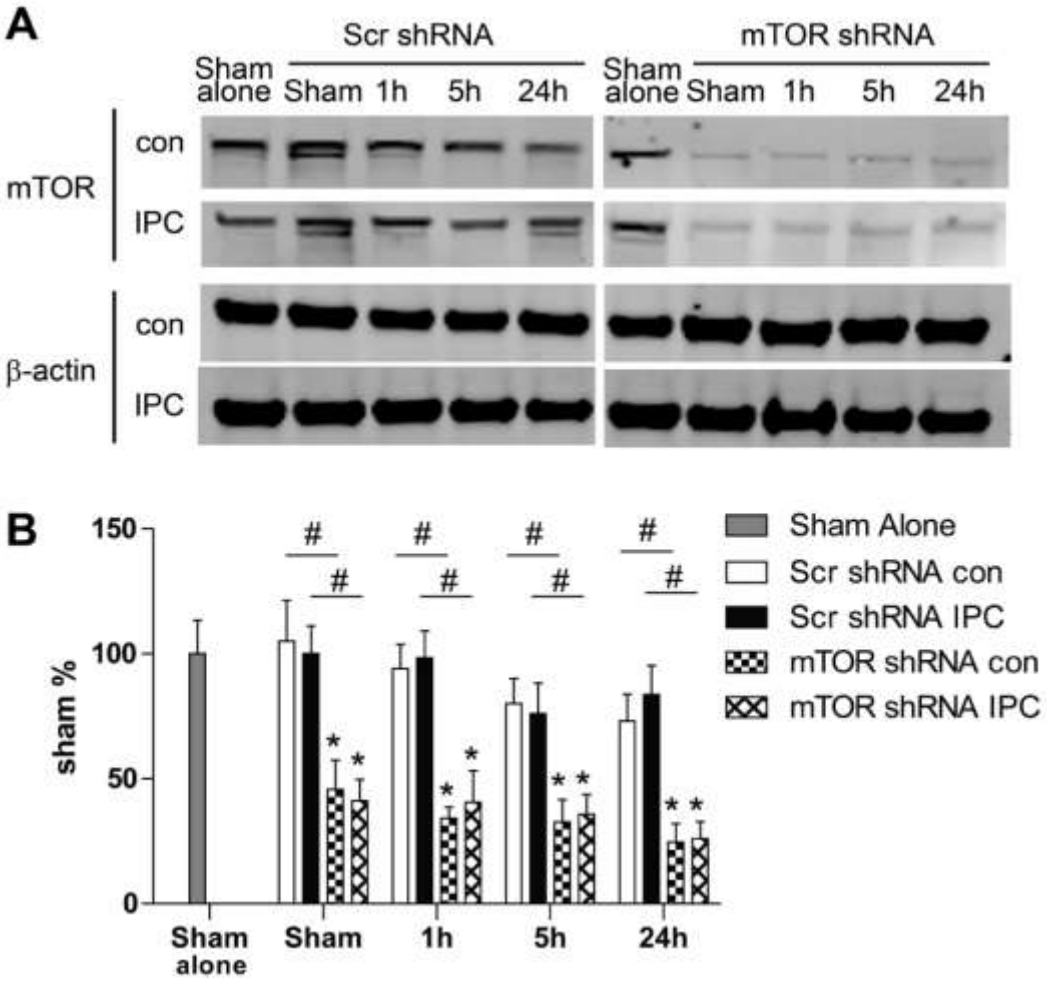
Supplementary Results



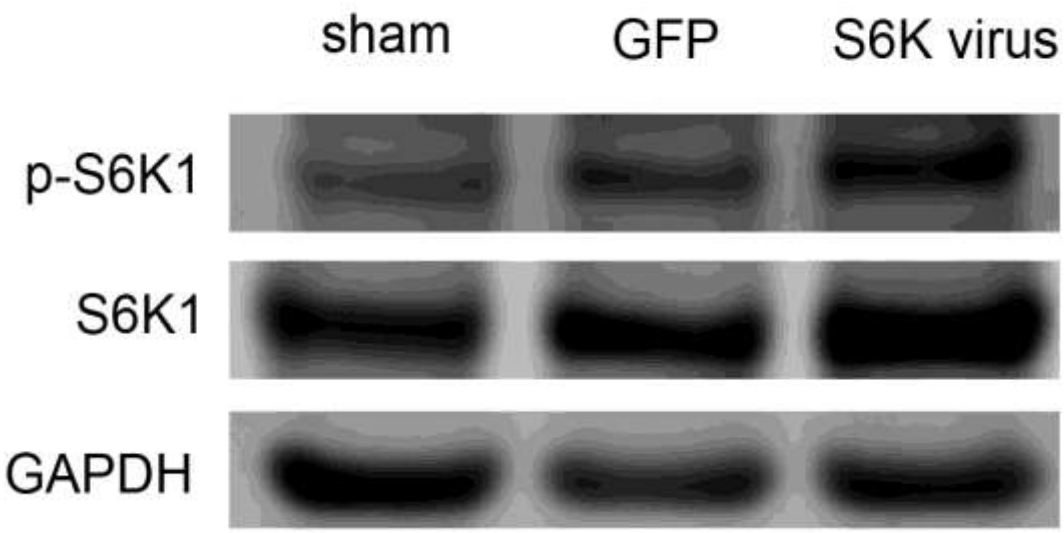
Supplemental Figure I. IPC did not alter total protein expression in the mTOR pathway. **A,** Representative protein bands of a Western blot for mTOR, S6K1, S6, and 4EBP1 at 1, 5, 9, and 24 hours post-stroke. β-actin indicates equal protein loading. **B,** Relative optical densities of protein bands normalized to those in the sham group and expressed as percentages. *, vs sham, $P<0.05$. n=8/group. Isc con, ischemic control; IPC, ischemic postconditioning.



Supplemental Figure II. Rapamycin did not alter total protein levels in the mTOR pathway. **A,** Representative protein bands of major proteins in the mTOR pathway. **B,** Quantified levels of each protein. *, vs. sham brain, $P<0.05$; &, vs. con (non-ischemic control with rapamycin injection), $P<0.05$. n=8/group. con, control; IPC, ischemic postconditioning; veh, vehicle; rapa, rapamycin.



Supplemental Figure III. The effects of mTOR shRNA on total protein levels in the mTOR pathway. **A,** Representative total protein bands in the mTOR pathway. Brain tissues from penumbra were collected and analyzed at 1, 5, 9, and 24 hours post-stroke with or without IPC. **B,** Bar graphs show quantified levels of each protein. *, vs. sham brain, $P<0.05$; # between the two indicated groups, $P<0.05$. n=8/group. Scr, scramble, con, control; IPC, ischemic postconditioning.



Supplemental Figure IV. Gene transfer of S6K virus promoted S6K1 and p-S6K1 protein expression. Lentiviruses were injected into the left cortex and brain tissues around the needle track were collected 5 days later. Proteins were extracted and Western blot was performed to determine protein levels of S6K1 and p-S6K1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a control for even protein loading.

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