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The mTOR cell signaling pathway is crucial to the long-term protective effects of ischemic postconditioning against stroke

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Abstract

Ischemic postconditioning (IPostC) protects against stroke, but few have studied the pathophysiological mechanisms of its long-term protective effects. Here, we investigated whether the mTOR pathway is involved in the long-term protective effects of IPostC. Stroke was induced in rats by distal middle cerebral artery occlusion (dMCAo) combined with 30 min of bilateral common carotid artery (CCA) occlusion, and IPostC was induced after the CCA release. Injury size and behavioral tests were measured up to 3 weeks post stroke. We used rapamycin and mTOR shRNA lentiviral vectors to inhibit mTOR activities, while S6K1 viral vectors, a main downstream mTOR gene, were used to promote mTOR activities. We found that rapamycin administration abolished the long-term protective effects of IPostC. In addition, IPostC promoted the presynaptic growth associated protein 43 (GAP-43) and the postsynaptic protein 95 (PSD-95) levels at 1 week post-stroke, which were reduced by rapamycin. Furthermore, rapamycin reduced phosphorylated mTOR (p-mTOR) protein levels measured at 3 weeks after stroke. These results were confirmed by mTOR shRNA transfection. Moreover, we found that injection of S6K1 viral vectors promoted GAP-43 and PSD-95 protein levels. We conclude that mTOR may play a crucial, protective role in brain damage after stroke and contribute to the protective effects of IPostC.

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Contributions of authors

Peng Wang participated in the creation of the animal model, behavior tests, running of western blots, and data analyses.

Rong Xie participated in the behavior tests, constructed the virus vector and participated in data analyses.

Michelle Cheng participated in the virus construction, and participated in data collection.

Robert Sapolsky participated in the experimental design and data analyses, and supervised the virus vector construction.

Xunming Ji participated in the concept formation and experimental design.

Heng Zhao participated in the experimental design, supervised the study, and drafted the manuscript.

Declarations of interest: none.

Keywords

focal cerebral ischemia; ischemic postconditioning; mTOR; stroke

Introduction

Ischemic postconditioning (IPostC), which refers to the interruption of reperfusion after stroke, protects against stroke induced brain injury [11–13, 21, 22, 25]. We and others have shown that IPostC improves glucose uptake [14], reduces free radical generation [25], inhibits inflammation [3, 11] and promotes protein activity in the PI3K/Akt pathway [5, 13, 17, 24]. Most recently, we demonstrated that the mTOR cell signaling pathway contributes to the acute protective effects of IPostC measured at 2 to 3 days after stroke. We found that IPostC attenuated reductions in phosphorylated protein levels after stroke in the mTOR pathway, including S6K1, S6, and 4EBP1. In addition, inhibition of mTOR, both by the mTOR inhibitor rapamycin and mTOR shRNA, worsened ischemic outcomes and abolished IPostC's protection, when measured at 2 to 3 days post stroke. Nevertheless, whether the mTOR pathway is involved in a long-term protective effect of IPostC has not been studied. In this present study, we evaluated the role of mTOR in long-term outcomes after stroke, with and without, IPostC. Bench markers used to evaluate the role of mTOR include brain injury size, behavioral tests, and their correlation with the expressions of various proteins in the mTOR pathway, along with presynaptic growth associated protein 43 (GAP-43) and postsynaptic density protein 95 (PSD-95).

Methods

Animals were housed under a 12-12 hour, light-dark cycle with food and water available, ad libitum. All experiments were conducted on Sprague-Dawley rats (Charles River, Wilmington, MA, USA) according to protocols approved by the Stanford Institutional Animal Care and Use Committee (IACUC) and NIH Guidelines for Care and Use of Laboratory Animals.

Construction, generation and titration of lentiviral vectors

We constructed lentiviral vectors that overexpress S6K1, a downstream protein of the mTOR pathway, to study the role of mTOR, as we previously reported [20]. The S6K1 gene was cloned from S6K1 (pcDNA3) plasmids (S6K1: 26610, Addgene, Cambridge, MA) and inserted into the lentiviral backbone plasmid, pHR⁺trIPostCMV-IRES-eGFP. The control vector was a lentiviral plasmid backbone with only eGFP inserted. We also constructed lentiviral vectors containing mTOR shRNA to inhibit the mTOR pathway, and a scramble shRNA gene as control (mTOR shRNA 1856, scramble shRNA 1864; Addgene, Cambridge, MA). The generation and titration of lentiviral vectors were detailed in our previous study [19]. Viruses were resuspended in phosphate-buffered saline (PBS) and kept at -80°C , and the virus titer range was adjusted from 1×10^8 – 5×10^8 TU/ml to 1×10^8 TU/ml by using PBS before gene transfer.

IPostC model, general histology, and infarct size measurement

We conducted focal cerebral ischemia and IPostC as previously described [4, 5, 14, 25]. Male Sprague-Dawley rats (300–350g) were anesthetized by 2–3% isoflurane. Body temperature was maintained at 37°C using a heat blanket, and monitored with a rectal probe. Stroke was induced by permanent dMCAo combined with bilateral CCA occlusion for 30 min. After the CCA was released, IPostC was performed by 3 cycles of 30 second CCA suture release followed by 10 seconds of occlusion [4, 5, 25]. Rats were euthanized at 3w after stroke and fixed with 4% paraformaldehyde (PFA) and 20% sucrose for 24h, then cut into 5 coronal blocks from rostral (level 1) to caudal (level 5) and stored at –80°C. Brain sections were stained with cresyl violet, and the average infarct size, based on all 5 section levels, were measured and calculated by a person who was blinded to the animal's group, and normalized to the contralateral cortex expressing as percentages to the ischemic hemisphere, as previously described [25, 26, 28].

Rapamycin and vectors injection in vivo

Rapamycin (Calbiochem, Billerica, MA, USA) was dissolved in PBS to a concentration of 1mM. PBS solution was used as control [20]; either 5µl of drug solution or vehicle (PBS) was infused into the ventricle ipsilateral to the ischemia 1h before ischemia, as described. [20]. Lentiviral vectors were injected 5d before ischemia as described [20]. 5µl of each viral vector: scramble shRNA, mTOR shRNA, GFP or S6K1 was injected at 0.5 µl/min into the cerebral cortex, ipsilateral to the ischemia (from bregma: anteroposterior, 0.96 mm; mediolateral, 3.5 mm; dorsoventral, 1.8 mm), and the needle was left for 10 min before being withdrawn.

Western blotting

Rats were euthanized at 1w and 3w after stroke (n = 8). A sham surgery group was used as the control. To study the effects of gene transfer on protein expressions in animals after stroke, brain tissues from a 1 mm diameter around the needle track were dissected from the peri-infarct region, and protein levels were detected by western blot [5, 26]. Rats were sacrificed at 1h, 5h, 9h, 24h, 1w and 3w after stroke (n = 8). Animals receiving sham surgery and the GFP virus vector, but without stroke, were prepared as control. Western blot was performed, as previously described, by using whole cell proteins extracted from the frozen brain tissue [5, 26, 27]. To conduct western blot, 20 µg of protein in each sample was subjected to SDS-PAGE using 4–15% Ready Gel (Cat: L050505A2; Bio-Rad, Hercules, CA). After protein bands were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), the membranes were incubated with primary antibodies overnight at 4°C followed by Alexa Fluor 488 donkey anti-rabbit or anti-mouse IgG secondary antibody (1:5000, Invitrogen, Eugene, OR, USA) for 1h in a dark room. Primary antibodies are listed in Table 1. Membranes were scanned using Typhoon Trio (GE Healthcare). The optical densities of all protein bands were analyzed using IMAGEQUANT 5.2 software (GE Healthcare).

Behavioral tests

All behavior tests were performed by a person blinded to the experimental groups. All tests were performed 1d before stroke surgery as a baseline and at 1d, 2d, 3d, 7d, 14d, and 21d after stroke. Neural deficits after stroke were quantified by using the vibrissae-elicited forelimb placing test and home cage limb test as previously described [26]. The vibrissae-elicited test was conducted by gently brushing the rat's vibrissae on each side against a table edge. The limb placement reflex was tested 10 times on each side per trial, and two trials occurred per test session. The percentage of vibrissae stimulations in which a paw placement occurred was calculated. After completing the former behavior test, the home cage test was performed. The rat was allowed to return to its home cage, and the number of times the rat used its forelimbs to brace itself against the cage wall was counted; the use of the ipsilateral, contralateral, or both forelimbs was counted separately, and a total of 20 contacts were counted. Percentages of the ipsilateral forelimb contact was calculated using this formula: $(\text{ipsilateral} + (\text{both} / 2)) / 20 \times 100\%$.

Statistical analysis

Statistical analyses were conducted by using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Infarct sizes were analyzed by one-way ANOVA followed by the Fisher's least significant difference (LSD) post hoc test. Western blot results were analyzed by using two-way ANOVA followed by the Fisher's LSD post hoc test. For behavioral tests, one-way repeated ANOVA was used to compare a test at different time points in the same group, and two-way ANOVA was used to compare tests between different groups, followed by the Fisher's LSD post hoc test. Tests were considered significant at P values < 0.05 . Data are presented as mean $\pm$ SEM.

Results

mTOR inhibition by rapamycin abolished the protective effects of IPostC against brain injury measured 3 weeks after stroke

Acute protective or detrimental effects of some neuroprotectants or neurotoxicants are not sustained over the long term, and infarct size may not always equate to neurological function. We therefore investigated whether inhibiting mTOR permanently affects brain injury and function. We previously reported that rapamycin injected before stroke resulted in larger infarction measured at 2d post stroke [20]. Nevertheless, rapamycin injection did not enlarge infarct size measured at 3w (21d) after stroke without IPostC (Fig. 1), but it did abolish the long-term protection of IPostC on injury size (Fig. 1). In both the home cage and vibrissae-induced forelimb placing tests, IPostC improved neurological scores (Fig. 2 A, B). While rapamycin did not worsen neurological deficits in animals receiving sham surgery or stroke alone (Fig. 2 C, D), it did reverse the beneficial effects of IPostC (Fig. 2 E, F).

mTOR inhibition by rapamycin and shRNA resulted in long-term changes in PSD-95, GAP-43 and p-mTOR protein expressions

Because mTOR activity has been linked to axon growth, synaptic plasticity, learning, and memory [7], we studied its effects on PSD-95 and GAP-43 protein levels (Fig 3A,B). These

two proteins were reduced at 1w after stroke, but recovered, to some extent, at 3w. With IPostC, their expression levels were increased at 1w. In animals with stroke alone, rapamycin had no effect on PSD-95 and GAP-43 protein levels, but in animals with stroke and IPostC, rapamycin significantly inhibited GAP43 levels, but not PSD-95, at 1w and 3w after stroke.

We also examined if early modulation of mTOR activity after stroke had long-term effects on p-mTOR protein levels (Fig. 3 C, D). Without IPostC, the protein levels decreased at 1w post-stroke, but recovered at 3w. With IPostC, their levels were significantly increased at 1w. Because rapamycin injection may produce systemic effects, we constructed mTOR-shRNA lentiviral vectors to confirm the effect of mTOR inhibition on the above protein levels. We showed that mTOR shRNA inhibited PSD-95 and GAP-43 protein levels in the ischemic brains with IPostC measured at both 1w and 3w post-stroke (Fig.4).

Gene transfer of S6K lentiviral vectors promoted protein expression of the functional proteins and S6K

To further confirm the important role of the mTOR pathway in long-term ischemic brain injury, we injected S6K lentiviral vectors into the ischemic penumbra. We have previously shown that S6K vectors inhibited infarct size measured 2d post-stroke and improved mTOR activity. Here, we further found that S6K promoted PSD-95 and GAP-43 protein levels measured at 24h, 1w and 3w after stroke (Fig.5). We also confirmed that a S6K virus injection did not alter mTOR levels, but enhanced S6K and its phosphorylation levels (Fig. 5).

Discussion

We recently reported that the mTOR cell signaling pathway contributes to the acute protective effects of IPostC against stroke. Using the same rat stroke model with IPostC, we now show that early mTOR modulation also affects the long-term recovery of brain function. First, we found that mTOR inhibition abolished the long-term protective effects of IPostC, as reflected by brain injury size measured at 3w post-stroke, and behavioral tests. Second, IPostC enhanced p-mTOR protein levels measured at 1w and 3w, but such effects were blocked by mTOR inhibition using both rapamycin and mTOR-shRNA. Third, IPostC improved functional PSD-95 and GAP-43 protein levels, which were inhibited by both rapamycin and mTOR-shRNA. Fourth, overexpression of S6K, the most critical mTOR downstream protein, promoted PSD-95 and GAP-43 protein levels. Taken together, mTOR activity plays an important role in long-term brain injury recovery.

It has been reported that the mTOR pathway is involved in brain injury induced by ischemia. However, whether it is beneficial or detrimental to the ischemic brain has remained controversial. mTOR activity increases when brain injury is protected by a number of neuroprotectants, including melatonin and estradiol [9, 10], tPA [18], and silibinin [16], suggesting that mTOR is beneficial to brain injury. In contrast, studies have also reported that mTOR is detrimental to neuronal survival, and that rapamycin attenuated ischemic cell damage [6] and protected against neuronal injury by autophagy induction after stroke. We recently provided strong evidence that mTOR is neuroprotective against stroke [20]. In our

previous study, we demonstrated that levels of multiple phosphorylated proteins in the mTOR pathway were reduced soon after stroke onset in the ischemic brain, including mTOR, S6K1, S6, 4eBP-1 and eIF4E, suggesting that the degradation of these proteins precedes final brain injury. However, IPostC attenuated these protein degradations. Second, we showed that both rapamycin and mTOR shRNA enlarged infarction, and that S6K1 gene transfer reduced neuronal death in vitro and inhibited infarction 2d post-stroke in vivo, further suggesting a neuroprotective role of mTOR in stroke. Although it is considered that the lentiviral vector driven by CMV promoter may mediate gene expression in glial cells, but few in neurons, our previous study did provide strong evidence that the lentiviral vectors strongly transfected neurons in both in vitro and in vivo [19], as shown by double staining of MAP-2 and GFP, as well as the neuronal morphology. Therefore, the lentiviral vectors may directly affect neuronal function rather than microglia. Nevertheless, we cannot exclude a possibility that the lentiviral vectors may also indirectly affect neuronal survival via their effects on microglia.

To understand the role of the mTOR pathway in short and long-term brain injury after IPostC, in our previous study, we first examined the acute effects of IPostC on the phosphorylated proteins in the mTOR pathway [20]. The results showed that IPostC attenuated degradations of phosphorylated mTOR S6K1, S6, 4eBP-1 and eIF4E in the acute phase after stroke. In addition, we showed that the administration of both rapamycin and mTOR shRNA enlarged infarction, and that S6K1 gene transfer reduced neuronal death in vitro and abolished the protective effects of IPostC measured 2d post-stroke in vivo. In this present study, we further demonstrated that acute mTOR inhibition executed long-term, detrimental effects on IPostC, as measured by protein expression and behavioral tests up to 3w after stroke. We showed that mTOR inhibition decreased neurological improvements afforded by IPostC for up to 3w, as shown by behavioral testing. The inhibitive effect of mTOR against IPostC correlated with attenuated p-mTOR protein levels.

Our results indicate that the protective effects of mTOR from IPostC may be due to its ability to promote synaptic plasticity and axonal growth, or neuronal structural stability in peri-infarct regions. To evaluate this, we measured GAP-43 and PSD-95 protein levels. GAP-43 expresses on presynaptic terminals, and is a known growth and plasticity protein [2, 15]. Its overexpression has been associated with improved brain recovery and learning ability following treatment with neuroprotectants after stroke [2, 15, 23]. PSD-95 is expressed on postsynaptic terminals and interacts with N-Methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptors and potassium channels to regulate neuronal activity [1, 8]. Although its acute interaction with glutamate receptors results in neuronal death [1], its expression in the late phase after stroke may be required for brain recovery. Our results show that IPostC increased GAP-43 and PSD-95 protein levels at 1w after stroke compared to control animals, but no difference was observed at 3w. Additionally, rapamycin inhibited GAP-43 in IPostC groups. We had some limitations in our study. First, our observations of the mTOR pathway and its regulations were limited to our IPostC model. This must be carefully considered when using our results to discuss the role of the mTOR pathway in stroke. Second, we failed to construct an mTOR virus, so a S6K lentivirus was used as an indirect proof. The mTOR pathway is an interesting target in ischemic stroke, needing further investigation in stroke research.

Conclusion

We have employed multiple approaches to inhibit and promote mTOR activity and measured their effects on long-term brain injury and neurological functions. We conclude that IPostC executes long-term protective effects against brain injury by promoting mTOR activity, which suggests mTOR as a potential target for neuroprotection in stroke treatment.

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List of Abbreviations

CCA	common carotid artery
dMCAo	distal middle cerebral artery occlusion
GAP-43	growth associated protein 43
IPostC	Ischemic postconditioning
mTOR	mammalian target of rapamycin
MCA	middle cerebral artery
PFA	paraformaldehyde
PSD-95	postsynaptic protein 95

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Highlights

- mTOR inhibition abolishes the long-term protection of postconditioning on injury size
- mTOR inhibition inhibited protein expressions linked with axon growth and synaptic plasticity
- mTOR promotion by S6K enhances protein expression linked with axon growth and synaptic plasticity

Fig. 1. The effects of mTOR inhibition by rapamycin on long-term brain injury size

Top: Representative brain sections stained with cresyl violet in rats euthanized 21d after stroke. Part of the brain tissue in the ischemic region was lost. Stroke resulted in cortical injury ipsilateral to the occluded MCA, whereas IPostC spared some of the injured cortex.

Bottom: Injury size measured 21d after stroke. IPostC significantly reduced injury size. Rapamycin did not increase infarct size in animals with stroke alone, but did abolish the protective effects of IPostC. #, $p < 0.05$, between the two indicated groups. $n = 8/\text{group}$. Isc, ischemia (control); IPC=IPostC, ischemic postconditioning (after reperfusion); rapa, rapamycin.

Fig. 2. The effects of mTOR inhibition by rapamycin on behavioral tests

Home cage and vibrissae tests were conducted from 0 to 21d after stroke. **Left column (A, C, E):** The home cage test suggests that rapamycin injection abolished the protective effects of IPostC. **A.** IPostC attenuated neurological deficits. * vs other groups at the same time point, $p < 0.05$; # vs the IPostC plus vehicle group at the same time point, $p < 0.05$. **C.** Rapamycin injection did not affect behavioral tests in animals with or without stroke. * The sham groups vs the other two groups at the same time point, $p < 0.05$; **E.** Rapamycin injection abolished the protective effects of IPostC on neurological deficits. * vs The sham groups vs the other two groups at the same time point, $p < 0.05$; #, vs isc + IPostC, $p < 0.05$. **Right column (B, D, F).** The effects of rapamycin injection on the vibrissae test results. **B.** IPostC attenuated contralateral forelimb placing deficits after stroke. * vs other two groups at the same time point, $p < 0.05$; and vs ischemia, $p < 0.05$; #, vs isc + IPostC, $p < 0.05$. **D.**

Rapamycin injection did not affect vibrissae tests in animals receiving stroke alone or sham surgery. * The sham group vs other two groups at the same time point, $p < 0.05$. **F.** Rapamycin injection worsened vibrissae tests in animals receiving stroke plus IPostC. * vs other two IPostC groups at the same time point, $p < 0.05$; and vs isc + IPostC+rapa, $p < 0.05$; # vs isc + IPostC, $p < 0.05$. $n = 8/\text{group}$. Isc, ischemia (control); IPC=IPostC, ischemic postconditioning (after reperfusion); rapa, rapamycin.

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Fig. 3. Long-term effects of rapamycin injection on PSD-95, GAP-43, and p-mTOR protein expression levels

A. Western blot shows representative protein bands of the neuron functional proteins, PSD-95 and GAP-43. GAPDH protein demonstrates even protein loading. **B.** Bar graphs show average protein optical densities, normalized to sham groups, and expressed as percentages. * vs sham, $p < 0.05$; #, between indicated two groups, $p < 0.05$. $n = 8$ /group. rapa, rapamycin; isc, ischemic; IPPostC, ischemic postconditioning. **C.** Western blot shows representative p-mTOR and mTOR protein bands. GAPDH protein demonstrates even protein loading. **D.** Bar graphs show average protein optical densities, normalized to sham

groups, and expressed as percentages. * vs sham, $p < 0.05$; #, between indicated two groups, $p < 0.05$. $n = 8/\text{group}$. rapa, rapamycin; isc, ischemic; IPC=IPostC, ischemic postconditioning.

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Fig. 4. Long-term effects on protein expression of mTOR-shRNA injection on PSD-95 and GAP-43, as well as p-mTOR protein levels

A. Western blot showing representative protein bands of the neuron functional proteins, PSD-95 and GAP-43. GAPDH protein demonstrates even protein loading. **B.** Bar graphs show average protein optical densities, normalized to sham groups, and expressed as percentages. * vs sham, $p < 0.05$; #, between indicated two groups, $p < 0.05$. $n = 8$ /group. isc, ischemic; IPostC, ischemic postconditioning. **C.** Western blot shows representative p-mTOR and mTOR protein bands. GAPDH protein demonstrates even protein loading. **D.** Bar graphs show average protein optical densities, normalized to sham groups, and

expressed as percentages. * vs sham, $p < 0.05$; #, between indicated two groups, $p < 0.05$. $n = 8/\text{group}$. isc, ischemic; IPC=IPostC, ischemic postconditioning.

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Fig. 5. Acute and long-term effects on protein expression of S6K virus injection on PSD-95 and GAP-43, as well as p-mTOR and P-S6K protein levels

A. Representative PSD-95 and GAP-43 protein bands. GAPDH protein demonstrates even protein loading. **B.** Bar graphs show average protein optical densities, normalized to sham groups, and expressed as percentages. * vs sham, $p < 0.05$; #, between indicated two groups, $p < 0.05$. $n = 8/\text{group}$. isc, ischemic; IPostC, ischemic postconditioning. **C.** Western blot shows representative p-mTOR, mTOR, p-S6K, and S6K protein bands. GAPDH protein demonstrates even protein loading. **D.** Bar graphs show average protein optical densities, normalized to sham groups, and expressed as percentages. * vs sham, $p < 0.05$; #, between

indicated two groups, $p < 0.05$. $n = 8/\text{group}$. isc, ischemic; IPostC, ischemic postconditioning.

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Table 1

Antibodies, concentrations and manufacturers used

Antibodies	Source	Dilutions	Manufacturer	Cat. #	Application
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
PSD	Rabbit	1:1000	Cell Signalling	2507	WB
GAP43	Rabbit	1:500	Cell Signaling	5307	WB