Rapamycin Pretreatment Alleviates Cerebral Ischemia/Reperfusion Injury in Dose-Response Manner Through Inhibition of the Autophagy and NFκB Pathways in Rats

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Abstract
Although rapamycin can attenuate cerebral ischemia/reperfusion (I/R) injury, the potential roles of rapamycin on cerebral I/R injury remain largely controversial. The present work aims to evaluate underlying molecular mechanisms of rapamycin pretreatment on I/R injury. In total, 34 Sprague-Dawley rats were randomly grouped to 3 groups: sham group (n = 2), vehicle group (n = 16), and rapamycin-pretreatment group (n = 16). Before the focal cerebral ischemia was induced, those rats in the pretreatment group were intraperitoneally injected rapamycin (1 mg/kg body) for 20 hours, while rats in the vehicle group received same-volume saline. Then, rats in these 2 groups received focal cerebral ischemia for 3 and 6 hours, respectively (n = 8 in each group), which was followed by the application of reperfusion for 4, 24, 72 hours, and 1 week (n = 2 in each group). The results showed that the rapamycin pretreatment improved the memory functions of rats after I/R injury, which was evaluated using a Y-maze test. Rapamycin pretreatment significantly reduced the size of triphenyltetrazolium chloride infarction and decreased the expression of I/R injury markers. Moreover, the expression of LC-3 and NFκB was also significantly reduced after rapamycin pretreatment. Taken together, rapamycin pretreatment may alleviate cerebral I/R injury partly through inhibiting autophagic activities and NFκB pathways in rats.

Keywords
rapamycin, dose-response, ischemia/reperfusion, stroke

Introduction
Stroke is accepted as the second leading cause of death in adults worldwide, which is characterized by cerebral ischemia because of disturbances for cerebral blood flow caused by thrombi or emboli. Despite advances in the treatment technique, the prognosis of stroke remains poor. Till date, very limited medications such as tissue plasminogen activator are used to treat ischemic stroke. However, they were reported to increase the risk of intracranial hemorrhage. Thus, there is clearly a need for investigating the underlying mechanism of stroke so as to develop new therapeutic strategies.

Rapid revascularization and reperfusion is reported to be powerful method for handling cerebral ischemia/reperfusion (I/R) damage. Unfortunately, the reperfusion usually evokes cerebral I/R injury, which is the most challenging complication for dealing with ischemic stroke. Ischemia/reperfusion causes the damage of blood–brain barrier (BBB) and neurons and releases various ions, chemicals, growth factors, and neurotransmitters, which further affect the BBB permeability and aggravate the neuronal damage during cerebra ischemia. Among the efforts that have been made to attenuate the I/R injury, numerous types of pretreatment measurements have been suggested by accumulating studies.

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Rapamycin as a macrolide antibiotic is originally used to be an antifungal agent in the 1970s.\textsuperscript{16,17} On the basis of its good immunosuppressed and antiproliferative characteristics, rapamycin was approved for immunotherapy of renal transplantation and even antitumor application.\textsuperscript{18,20} Recently, due to the reported antiapoptotic and autophagy-stimulating capacity, rapamycin has been demonstrated to decrease neuronal damage following traumatic brain injury, tuberous sclerosis, and Alzheimer disease.\textsuperscript{16,21} Moreover, studies reported that rapamycin attenuated cerebral I/R injury. However, the potential roles of rapamycin pretreatment on I/R injury together with underlying mechanisms remain largely controversial.\textsuperscript{16,17,21,22} The objective of this work was to assess the effectiveness of rapamycin pretreatment for rats with cerebral I/R injury.

**Materials and Methods**

**Animals**

The research was approved by the ethics committee for Shanghai Fengxian District Central Hospital research group. All experimental procedures were conducted strictly on the basis of the Guidelines for Animal Experimentation of Shanghai Fengxian District Central Hospital research group. Male Sprague-Dawley (SD) rats (age 10-12 weeks; weighed 280-320 g) were purchased from Cavins. Rats were housed by controlled conditions (12-hour light/dark cycle, 21 °C ± 2 °C and 60%-70% humidity) and can freely forage and have access to tap water.

**Experiment Protocol**

In total, 85 SD rats were randomly grouped into 3 groups: sham group (n = 5), vehicle group (n = 40), and rapamycin-pretreatment group. Before the focal cerebral ischemia was induced, those rats in the pretreatment group were intraperitoneally injected rapamycin (1 mg/kg body) for 20 hours while rats in the vehicle group received same-volume saline. Then, rats in these 2 groups received focal cerebral ischemia for 3 and 6 hours, respectively (n = 20 in each group), which was followed by the application of reperfusion for 4, 24, 72 hours, and 1 week (n = 5 in each group).

**Establishment of Rat I/R Models**

The rat focal I/R models were established through middle cerebral artery occlusion (MCAO). Rats were anesthetized using sodium pentobarbital (40 mg/kg; Sigma Aldrich, St. Louis, Missouri) intraperitoneally. The incision was made on the midline of the neck, and the right common carotid artery (CCA), internal carotid artery (ICA), and the external carotid artery (ECA) were isolated using a 6.0 silk suture. The micro aneurysm clips around the CCA and CEA was then placed for the prevention of bleeding, followed by the insertion of a needle (2-mL injector). After that, the rounded tips of 4.0 monofilament nylon threads (Ethicon, Johnson and Johnson) for MCAO were made through heating before using. We tightened loose collar sutures and finished the MCAO. The length of MCAO time was decided upon the experiment protocol. After the conduction of MCAO, the reperfusion was undertaken using the nylon filament.

**Y-Maze Test**

Four hours after surgery, rats were placed in Y-type labyrinth box in a dark and calm environment for 5 minutes. After that, the arms of the Y-type labyrinth box and the other arm were energized (voltage 70 V, 2-second delayed electric shock, 15-second continued light, 45-second each training). Taking the arm where the animal was in as the starting position for the next test, we set the order of I → II → III → I as a safety zone. We continuously trained the rats following the protocol introduced above until they successfully learned it 1 day. The “correct response” was defined as rats rushed to the safe area within 10 seconds after electrification stimulation, otherwise it was defined as a “false response.” Successful training was defined as 9 or more correct responses of the 10-consecutive stimulation. The number of electric shocks used to meet the successful training standard were recorded to evaluate the ability of learning and spatial resolution. After 24 hours of learning tests, the same method was used to test 10 more times, to correctly evaluate the memory retention ability of each rat.

**Evaluation of Infarct Volume**

Following reperfusion for 4, 24, 72 hours, and 1 week, respectively, rats were sacrificed and their brain tissues were collected. Then, about 3-mm-thick sections were obtained and stained by triphenyltetrazolium chloride (TTC). After 30 minutes, they were transferred into 4% paraformaldehyde solution for 48 hours. The infarcted regions were white, while the normal tissues were red. Infarcted and non-infarcted regions were analyzed and visualized by Image-ProPlus software. Total infarct volume of each sample studied was computed. The ratio of the infarct volumes was also calculated to evaluate and compare the cerebral infarction between the groups.

**Immunofluorescence Staining**

For the immunofluorescence (IF) staining of brain tissue slides, 0.05% Triton X-100 and 5% bovine serum albumin (BSA) for 1 hour was added into slides for blocking. The incubation of coverslips was completed by primary antibodies: Aβ (1:500, ab59592; Abcam), BACE1 (1:100, PR-1661, Hope), CTSB (1:100, bs-1500R, bios), Caspase-3 (1:1000, ab49822; Abcam), NFκB (1:500, ab16502; Abcam), and LC-3 (1:500, ab192890; Abcam) at 4 °C for 24 hours. Then, fluorescence-conjugated secondary antibodies (GAM4882; MULTI SCIENCES) were added and nuclei were stained using DAPI (Beyotime) for 5 minutes.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction**

Total RNAs were obtained by TRizol reagent kit (Takara). Then, complementary DNA was synthesized with PrimeScript
RT-PCR kit (Takara). The real-time polymerase chain reaction (RT-PCR) was conducted with the SYBR Green PCR Master Mix on the ABI 7900 Prism HT (Applied Biosystems). Primers used are as followed: Aβ, forward: GGGCTCGTCAGTTTCCCT; reverse: GTCATCTCCTCCGCATCA; BACE-1, forward: TCTAGGTGTGTTAGGAGGTCT; reverse: GTCGGCAGCCCATATTGGAACTSB; forward: TTTG GCACGTCTCCTGTCT; reverse: TGTCATGTTCACTGTCGGCCA; Caspase-3, forward: TGGTTGAGGTTGAGGC; reverse: AAACGTCTCGTTGCCTGT; NFκB, forward: CTCGTCAGTCTGTTGGTT; reverse: TCGTCTGCCTGGCGAAAAAT; LC-3, forward: ; reverse:; GAPDH, forward: GGCACAGTCAAGGCTGAGAATG; reverse: ATGG TGGTGAAGACGCCAGTA. Finally, the messenger RNA (mRNA) expression between the groups was determined by $2^{-\Delta\Delta Ct}$ method.

**Western Blotting**

Following reperfusion for 4, 24, 72 hours, and 1 week, respectively, total protein of the brain tissue was extracted using radioimmunoprecipitation assay. The total concentration of target protein was measured using a spectrophotometer. The proteins (60 g) were separated by 10% sodium dodecyl sulfate polyacrylamide gels. After that, they were transferred onto polyvinylidene difluoride membranes (Shanghai Haoran Bio Technology Co, Ltd) and blocked with 5% BSA for 2 hours, followed by incubation with primary antibodies specific to Aβ (1:500, ab59592; Abcam), BACE1 (Hope, PR-1661, 1:300a), CTSB (bioss, bs-1500R, 1:500), Caspase-3 (1:500, ab49822; Abcam), NFκB (1:100, ab192890; Abcam) and LC-3 (1:100, ab192890; Abcam) for 24 hours at 4°C. And then, a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, ZSBIO, ZB-5305) was added for 2 hours at 25°C. Immunoblotting was undertaken by enhanced chemiluminescent autoradiography (Bioroc Pharmaceutical & Biotech Co, Ltd). Finally, the protein was quantified by the Bio-Rad system (Bio-Rad).

**Statistical Analysis**

All assays were carried out repeatedly 3 times. Statistical analyses were conducted by 1 way analysis of variance. The Student-Newman-Keul post hoc analyses and Student 2-tailed t test were performed using SPSS version 15.0. The data were exhibited as mean ± standard deviation. Herein, the $P < .05$ was a cutoff of statistical significance.

**Results**

**Alterations of Neurological Functions**

The results of Y-maze test showed that ischemia significantly reduced the neurological functions of rats. The number of correct response in I/R group was remarkably decreased compared with the sham group. In addition, as the time of ischemia got longer, the savageness of neurological function deficit got worse. When compared with the I/R group, rapamycin-pretreatment group significantly increased the memory functions of rats after I/R injury, regardless of the time of reperfusion for 4, 24, 72 hours, or 1 week (Figure 1).

**Cerebral Infarct Volume**

The results of TTC staining showed that obvious infarct area was in the tissue samples of I/R group, while there was no infarct volume in sham groups. As the time of ischemia get longer, the infarct volume of the brain tissue increased significantly. However, the length of reperfusion was negatively correlated with the infarct area Rapamycin pretreatment at the same time point significantly reduced the infarct volume in groups with I/R injury compared to the I/R group, regardless of the time of reperfusion for 4, 24, 72 hours, or 1 week (Figure 1).

**Histological Changes in Brain Tissues**

The results of IF showed that I/R injury dramatically elevated levels of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 when compared with samples from the sham group, the expression of which gradually increased when the time of ischemia get longer. The length of reperfusion was also found to be negatively correlated with the expression of the markers mentioned above. The levels of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 were markedly reduced in rapamycin-pretreatment groups with I/R injury compared with control group, regardless of the time of reperfusion for 4, 24, 72 hours, or 1 week (Figure 2).

**Alterations of mRNA Levels of Aβ, BACE1, CTSB, CASPASE-3, NFκB, and LC-3**

The results of RT-PCR showed that I/R injury significantly increased the mRNA expression of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 when compared with samples from the sham group, the expression of which gradually increased when the time of ischemia get longer. The length of reperfusion was also found to be negatively correlated with the mRNA expression of the markers mentioned above. Rapamycin pretreatment at the same time point significantly reduced the mRNA expression of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 after I/R injury (Figure 3).

**Alterations of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 Protein Expression**

Being similar to the results of IF, the results of Western blot analysis showed that I/R injury remarkably enhanced the levels of Aβ, BACE1, CTSB, Caspase-3, NFκB, and LC-3 when compared with samples from the sham group, the expression of which gradually increased when the time of ischemia get longer. The length of reperfusion was also found to be negatively correlated with the expression of the markers mentioned above. There was a significant reduction of Aβ, BACE1,
CTSB, Caspase-3, NFκB, and LC-3 after I/R injury in rapamycin-pretreatment group at the same time point than I/R group, regardless of the time of reperfusion for 4, 24, 72 hours, or 1 week (Figure 4). These results indicated that rapamycin pretreatment inhibited both the neurological damage and the activation of inflammatory and autophagy pathways.

**Discussion**

In this article, we demonstrated that rapamycin, a macrolide antibiotic originally used as antifungal agent, could act as a cerebral I/R injury suppressor, as evidenced by its improvement of neurological functions and the decrease in the cerebral infarct volume after cerebral I/R injury. In addition, rapamycin pretreatment decreased both the mRNA and protein level of neurological damage markers, including Aβ, BACE1, CTSB, and Caspase-3. We further found that the levels of NFκB and LC-3 also dramatically reduced after the pretreatment of rapamycin before I/R injury. These findings indicate that rapamycin pretreatment alleviates cerebral I/R injury, probably partly by inhibiting the autophagic activities and NFκB pathways in rats.

The severity of I/R injury is mainly determined by the time length of ischemic.

In this study, we induced cerebral ischemic at a duration of 3 and 6 hours, respectively. As is expected, the severity of cerebral I/R injury in the longer time (eg, 6 hours) was significantly worse than that of the shorter time (eg, 3 hours). Numerous studies showed that time limit of reperfusion has been proved to play key roles in determining the damage degree of cerebral ischemic. In this study, we set the time limits of reperfusion as 4, 24, 72 hours, and 1 week. Interestingly, the results showed that prolonging the time limits of reperfusion from 4 hours to 4 weeks benefits the recovery of neurological functions after cerebral I/R injury.

The underlying effects of apoptosis and inflammation in I/R injury have been established by previous studies. It has been shown that the expression of inflammation and apoptosis precursors including Aβ, BACE1, CTSB, and Caspase-3 has been increased in rodents with focal cerebral ischemia and cerebral I/R injury, especially due to the disruption of

Figure 1. Rapamycin pretreatment significantly reduced the infarct volume of brain after I/R injury. I: ischemia; R: reperfusion; Rapa: rapamycin pretreatment.
Figure 2. The results of immunofluorescence staining showed that rapamycin pretreatment significantly reduced the protein expression of Aβ, BACE1, Caspase-3, CTSB, Caspase-3, NF-κB, LC3. I: ischemia; R: reperfusion; Rapa: rapamycin pretreatment.
Figure 3. The results of RT-PCR analysis showed that rapamycin pretreatment significantly reduced the mRNA level of Aβ, BACE1, CTSB, Caspase-3, NF-κB, LC-3. I: ischemia; R: reperfusion; Rapa: rapamycin pretreatment.
Inhibition of NFκB signaling has been proved to alleviate inflammatory disease conditions, including cerebral I/R injury. This may partly explain the neuroprotection of rapamycin for I/R injury. Additionally, autophagy has also been previously reported to have important roles in the pathophysiology of cerebral I/R injury. There are studies reporting the mechanism for the neuroprotective effects of rapamycin when microglial activation was inhibited and...
autophagy activity was reduced.\textsuperscript{34,35} Other studies showed that to avoid the overload of calcium and maintain homeostasis were the 2 main causes for rapamycin protection against I/R injury.\textsuperscript{36} Our results demonstrated that rapamycin pretreatment markedly reduced mRNA and protein levels of LC-3, which may indicate the inhibition effect of autophagy by rapamycin pretreatment. However, as a major limitation, this was an in vivo study without further in vitro confirmation. The expression changes of the subtype of LC-3 still remained to be elucidated. Thus, further studies are needed.

**Conclusion**

In conclusion, rapamycin pretreatment may alleviate cerebral I/R injury partly by inhibiting the autophagic activities and NF-kB pathways in rats.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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