Temporal and Spatial Expression Characteristics of MiR-155 and Rheb/mTOR Signaling Pathway in Ischemia–Reperfusion Injury of Rats

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Abstract

Backgrounds: Stroke is the second most prevalent cause of death and the first cause of longterm disability worldwide. Inhibition of miR-155 was found playing a protective role in ischemic stroke, one possible mechanism was regulating Ras-homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway. For possible specific intervention strategy, further exploring the expression characteristics of miR-155 and mRNAs of the Rheb/mTOR pathway in ischemic stroke is necessary.

Results: Our results demonstrated that the infarction volume decreased with the prolongation of the reperfusion in the MCAO/R model rats ($P < 0.05$). Meanwhile, the miR155 expression obviously increased in both the ischemic core and the ischemic penumbra (IP) area of the model rats, but this trend weakened as the reperfusion time increased. Besides, the expression of mRNAs of Rheb, mTOR, S6kb1, and 4Ebp1 seemed to increase in both the ischemic core and the IP area of the model rats. Interestingly, the mRNA level of S6kb1 obviously increased of all model groups in both the ischemic core and the IP area ($P < 0.05$), while the mRNA levels of Rheb, mTOR, and 4Ebp1 increased in the first 24 h and rapidly decreased after 48 h and as a result, a statistically significant difference was found only in the 48-h group ($P < 0.05$).

Conclusion: Along with the shrunk infarct volume, the levels of miR-155 decreased and the S6kb1 mRNA level increased as the lightening of re-perfusion, as to the mRNA levels of Rheb, mTOR, and 4Ebp1, statistical significance was found only in the 48-h group. Unexpectedly, there was no difference between the ischemic core and the IP area for all the above molecules. Indicating that intervention measures targeting to miR155 should be taken systemically as early as possible after stroke onset, especially within the early 48 hours.

Introduction

Stroke is the second most prevalent cause of death and the most common cause of long-term disability worldwide[1]. It is classified into two types: ischemic stroke (IS) and hemorrhagic stroke. In brief, IS is the general call of cerebral tissue necrosis and neurocyte apoptosis caused by insufficient blood supply due to stenosis or occlusion of the artery responsible for blood supply, which accounts for almost 80% of all strokes[2]. If no effective treatment is adopted in time, the cellular adenosine triphosphate (ATP) in storage is exhausted, further affecting various cellular physiological processes depending on ATP, such as depolarization of cell membrane, function of ion channels, intake of nutrients, and so forth. This leads to the release of excitatory neurotransmitters, activation of phospholipase, production of radicals, overload of intracellular calcium, activation of inflammatory factors, and so forth, resulting in irreversible cerebral injury and neurocyte death[3]. Thus, effective measures to alleviate the extent of infarction and its subsequent disability are essential[4, 5]. At present, two types of noninvasive clinical therapies for IS are offered: rebuilding of blood flow and neuroprotection. Systemic thrombolysis using recombinant tissue-type plasminogen activator is the only therapy believed to be effective and approved by the US Food and Drug Administration(FDA) for treating patients with IS. However, no more than 2% of patients with IS could benefit from thrombolysis therapy because of the narrow time window (within 3–4.5 h of IS

onset), increased risk of hemorrhagic complications, and ischemia–reperfusion damage[6]. Neuroprotection therapy can be used alone or together with thrombolysis therapy, which is primarily aimed to rescue the brain tissue of the boundary zone of the ischemic core, also called ischemic penumbra (IP)[7]. Nevertheless, a majority of neuroprotective agents show poor effects or serious toxicity/side effects in clinical trials as no neuroprotective agents of definitely curative effect are used in clinics at present[8]. Given the very limited treatment approaches for IS, new therapeutic strategies for this devastating disease need to be urgently developed. Many molecular mechanisms, such as inflammation, production of massive active oxygen, and excessive activation of autophagy, attribute to cerebral ischemia–reperfusion injury[3]. One of these is based on the ability of the mTOR pathway to prevent the apoptosis of neuronal cells, inhibit cell death due to excessive autophagy, promote neurogenesis, and accelerate angiogenesis. Therefore, mTOR may have the potential of preventing ischemic neuronal death and promoting neurological recovery. Thus, it may be a promising intervention target for IS[9].

MTOR was first proved and cloned in 1933 by Brown and his colleagues[10]. It is an atypical serine/threonine protein kinase belonging to the phosphatidylinositol kinase–related kinase protein family whose molecular weight is 289 kDa. It is highly conserved among species and plays important roles in regulating cell growth, proliferation, apoptosis, autophagy, and cell cycle. Rheb is a necessary protein in mTOR's activation[11, 12]. The downstream target proteins of mTOR primarily include ribosomal protein S6 kinase1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1). Activated mTOR regulates translation initiation and cell growth by phosphorylating to activate these downstream target proteins. Activated S6K1 promotes mRNA production, ribosomal protein translation, and cell growth[13]. Hypophosphorylated 4EBP1 binds to eIF4E through eIF4 gamma (eIF4G), a protein that helps transport mRNA to the ribosomes and further blocks protein translation, while phosphorylation induces the 4EBP1 dissociating from eIF4E, leading to eIF4G mRNA translation[14].

Enhanced activity of mTOR is known to inhibit autophagy, whereas mTOR inhibition promotes autophagy [15]. Further, mTOR inhibitors or mTOR gene silencers have been reported to induce cell death due to autophagy[16–20]. Previous studies have indicated that autophagy shows dual effects in the survival of neuronal cells after IS. The level of autophagy is crucial to its effect in cerebral ischemia. Physiological degrees of autophagy are beneficial to the survival of neural cells, but inadequate or excessive degrees can be detrimental and result in injury[21]. Our previous study in rat MCAO/R model also demonstrated that the activity of mTOR signaling pathway was inhibited while autophagy was activated during brain ischemia–reperfusion injury (unpublished, please see the complementary data).

MicroRNA (miRNA) is a series of long-chain noncoding RNA with a length of about 20–22 bp of nucleotides. It is wide-acting and has high regulation efficacy, and hence gained attention of researchers working on various diseases. MiRNAs are highly conserved among various eukaryotes [22]. MiRNAs and their corresponding target mRNAs have complementary pairing ending noncoding regions. Thus, the target mRNA molecules are degraded or translationally inhibited after pairing with the miRNAs, thereby further participating in the regulation of cell growth, differentiation, energy metabolism, apoptosis, and other important and basic cellular physiological processes.[23]. One miRNA may have multiple target

mRNAs, and an mRNA can be regulated by several miRNAs. [24]. MicroRNA155 (MiRNA-155) is a member of the miRNA family having all characteristics of miRNA. It has been proved to be involved in many diseases such as cancer, inflammation, and myocardial infarction and development [25]. A recent study demonstrated that miR155 inhibited the activity of the mTOR signaling pathway under hypoxic conditions by targeting the mRNA of multiple molecules, including Rheb, Ribosomal Protein S6 Kinase polypeptide 2(RPS6KB2), Rapamycin insensitive companion of mTOR (RICTOR), and so forth, in the mTOR signaling pathway. This led to their degradation or translation inhibition, and further induced the autophagy process and promoted the apoptosis of cells, indicating that miR155 might be involved in the pathology of cerebral ischemic damage [26]. Similarly, Guo Ping Xing et al. reported that the inhibition of miR-155 might play a protective role in IS by avoiding the action on Rheb mRNA and further phosphorylating S6K via the Rheb/mTOR pathway[27]. However, they did not explore whether any differences existed between the ischemic core and the boundary zone. Besides, they chose only one unstated time point of the ischemic process to test the aforementioned molecules in translational levels rather than posttranscriptional levels. Exploring the temporal and spatial expression characteristics of the miR-155 and the Rheb/mTOR pathway for the potential intervention project of the IS is necessary and essential. This study focused on and explored this problem, laying a foundation for further investigation of the relative intervention strategy.

Result

TTC staining results of brain tissues at different reperfusion times

The infarction volume of coronal sliced brain tissues of the MCAO/R model rats at different reperfusion times was determined by TTC staining to assess the potential injury content of the brains of MCAO/R rats at different reperfusion times. Figure 1A shows that the normal brain tissues were stained red whereas the infarcted tissues were stained white. The reperfusion times of different groups were marked on the top of the images, and slices of different brain levels were presented. Obviously, all the model groups exhibited infarcted tissues, but the sham groups did not. Quantitative assays of the relative infarction volume ratio at different reperfusion times were calculated to explore the correlation between the infarction volume and the reperfusion time, which has been demonstrated with a column chart in Fig. 1B. Further analysis of the average infarction volume ratio between different groups was conducted. All MCAO/R model rats exhibited increased infarction volume compared with their time-matched sham counterparts (Figure. 1B; $*P < 0.05$). Furthermore, the relative infarction volume ratio reduced as the reperfusion time increased (Figure. 1B; $\# \& P < 0.05$), in a time-dependent manner.

miR-155 levels in ischemic core and the boundary zone of model rats at different reperfusion times

After ischemia for 90 min, both the ischemic core and the boundary zone of the brain tissues of MCAO/R rats were collected dynamically 24, 48, and 72 h after reperfusion to see the characteristics of miR-155 expression in the ischemic brain. Then, the total miR-155 levels were detected by qRT-PCR as described earlier. The specific miR-155 levels of different groups and areas were calculated, and are depicted as a column chart (Figure. 2A) and a line chart (Figure. 2B) after being standardized using inner reference U6. The miR-155 level in the model rats was substantially higher than that in the time-matched sham group both in the ischemic core and the boundary zone (Figure. 2A, $P < 0.05$), and the tendency seemed to be less obvious as the reperfusion time prolonged. However, 72 h after reperfusion, the statistical difference compared with the sham group still existed. In addition, miR-155 levels in the ischemic core area were slightly higher than those in the boundary area, although no statistical difference was found ($P > 0.05$). Interestingly, the miR-155 level was the highest in the boundary zone 24 h after reperfusion, while it remained at a much higher level in the ischemic core both 24 and 48 h after reperfusion. These results suggested that the levels of miR-155 were upregulated in the brain tissues after ischemia–reperfusion injury, which was in line with several previous findings that hypoxia induced miR-155 upregulation [26], or miR-155 expression was significantly higher in rat MCAO/R model than in the control group [27], indicating that miR-155 might be involved in ischemic injury.

Rheb mRNA levels in the ischemic core and the boundary zone of model rats at different reperfusion times

After ischemia for 90 min, both the ischemic core and the boundary zone of the brain tissues of MCAO/R rats were collected dynamically 24, 48, and 72 h after reperfusion to see the characteristics of Rheb mRNA expression in the ischemic brain. Then, the total mRNA was extracted, and Rheb mRNA was tested by the qRT-PCR method described earlier after reverse transcription by the poly-A tailing method. The data were analyzed, and were depicted as a column chart (Figure. 3A) and a line chart (Figure. 3B) after being standardized using GAPDH. Compared with the time- and area-matched sham group, the Rheb mRNA level in the model group first increased till 48 h, reached the top, and then decreased. It was substantially higher in both the ischemic core ($^{\#}P < 0.05$) and the boundary zone ($^{*}P < 0.05$) 48 h after reperfusion. However, although it remained at a slightly higher level after 24 and 72 h of reperfusion in both the boundary zone and the ischemic core, no statistically significant difference was observed ($P > 0.05$).

The mTOR mRNA levels in ischemic core and the boundary zone of model rats at different re-perfusion time

After ischemia for 90 min, both the ischemic core and the boundary zone of the brain tissues of MCAO/R rats were collected dynamically 24, 48, and 72 h after reperfusion to see the characteristics of mTOR mRNA expression in the ischemic brain. Then, the total mRNA was extracted, and mTOR mRNA was tested by the qRT-PCR method described earlier after reverse transcription by the poly-A tailing method. The data were analyzed, and depicted as a column chart (Figure. 4A) and a line chart (Figure. 4B) after

being standardized using GAPDH. Compared with the time- and area-matched sham group, the mTOR mRNA level in the model group first increased till 48 h, reached the top, and then decreased. It was substantially higher than that in the sham group both in the ischemic core ($^{\#}P < 0.05$) and in the boundary zone ($^*P < 0.05$) after 48 h, as well as in the boundary zone after 72 h of reperfusion ($^{\&}P < 0.05$). However, although it remained at a slightly higher level after 24 h of reperfusion in both the boundary zone and the ischemic core, no statistically significant difference was observed after 72 h of reperfusion in the ischemic core ($P > 0.05$).

S6kb1 mRNA levels in the ischemic core and the boundary zone of model rats at different reperfusion times

After ischemia for 90 min, both the ischemic core and the boundary zone of the brain tissues of MCAO/R rats were collected dynamically 24, 48, and 72 h after reperfusion to see the characteristics of S6kb1 mRNA expression in the ischemic brain. Then, the total mRNA was extracted, and the S6kb1 mRNA was tested by the qRT-PCR method described earlier after reverse transcription by the poly-A tailing method. The data were analyzed and depicted as a column chart (Figure. 5A) and a line chart (Figure. 5B) after being standardized using GAPDH. Compared with the time- and area-matched sham group, the S6kb1 mRNA level in the model group was substantially higher in the ischemic core ($^{\#}P < 0.05$) of different reperfusion groups; it first increased till 48 h, reached the top ($^*P < 0.05$), and then rapidly decreased in the boundary zone. It nearly reached the normal level 72 h after reperfusion ($P > 0.05$).

4Ebp1 mRNA levels in the ischemic core and the boundary zone of model rats at different reperfusion times

After ischemia for 90 min, both the ischemic core and the boundary zone of the brain tissues of MCAO/R rats were collected dynamically 24, 48, and 72 h after reperfusion to see the characteristics of 4Ebp1 mRNA expression in the ischemic brain. Then, the total mRNA was extracted, and the 4Ebp1 mRNA was tested by the qRT-PCR method described earlier after reverse transcription by the poly-A tailing method. The data were analyzed and depicted as a column chart (Figure. 6A) and a line chart (Figure. 6B) after being standardized using GAPDH. Compared with the time- and area-matched sham group, the 4Ebp1 mRNA level in the model group first increased till 48 h, reached the top, and then decreased. It was substantially higher both in the ischemic core ($^{\#}P < 0.05$) and in the boundary zone ($^*P < 0.05$) 48 h after reperfusion. However, although it remained at a slightly higher level 24 and 72 h after reperfusion in both the boundary zone and the ischemic core, no statistically significant difference was observed ($P > 0.05$).

Discussion

In the present study, the infarction volume in the MCAO/R model rats decreased with the prolongation of the reperfusion time ($P < 0.05$). Meanwhile, the miR155 expression obviously increased in both the

ischemic core and the IP area of the model rats, but this trend weakened as the reperfusion time increased. Meanwhile, the expression of mRNAs of key molecules in the Rheb/mTOR signaling pathway, including Rheb, mTOR, S6kb1, and 4Ebp1, seemed to increase in both the ischemic core and the IP area of the model rats, but statistically significant difference was observed only in the 48-h group ($P < 0.05$). Interestingly, the mRNA expression level of S6kb1 obviously increased in all the 24-, 48-, and 72-h groups in both the ischemic core and the IP area ($P < 0.05$). In general, no obvious difference was observed in the expression characteristics of these molecules between the ischemic core and the IP area ($P > 0.05$), indirectly indicating that miR-155 participated in the ischemia–reperfusion injury; however, it showed a significant difference between the groups of different reperfusion times.

IS constitutes almost 80% of the stroke cases, while the remaining 20% are hemorrhage strokes [29]. Once blood flow to the brain is insufficient, the cells experience a series of molecular programs, including the toxicity of excitatory neurotransmitters, dysfunction of mitochondria, acidosis, imbalance of ions, oxidative stress, and inflammation. These molecular programs can result in cell death and nonreciprocal tissue damage[30]. Oxidative stress in the brain results in ischemic injury [31, 32]can eventually initiating programmed cell death pathways, including apoptosis, autophagy, and necroptosis [33–35]. With the wide application of intravenous thrombolysis and arterial embolectomy therapy in clinics at present, blood rebuilding is no doubt the most effective treatment for IS, which has been widely proved by clinical and experimental studies. This is also what exactly the MCAO/R model is based on and widely used in IS studies *in vivo*. Thus, the longer the blood rebuilding, the lighter the ischemic injury, as demonstrated in the present study (Figure. 1).

Specific stroke-induced miRNA expression profiles have been reported in both the blood and the brain, and in experimental models and patients at different reperfusion times[36–38]. The pattern of circulating miRNA expression suggests an early influence of age in stroke pathology, with a later emergence of sex as a factor for stroke severity [39]. Changed inflammation-related microRNA profiles in plasma following IS have been reported [40]. In addition, the patterns of miRNA expression were used to predict stroke subtypes [38]. MiRNA 155 has been demonstrated to mainly participate in neuroinflammation [41]. Although previous studies have investigated miRNA155 expression in ischemic animal models, only a few studies have focused on miRNA 155 in the IS. Xx et al. demonstrated that miR155 potently induced autophagy under hypoxic conditions by targeting multiple mRNA molecules of the mTOR signaling pathway, thus accelerating cell apoptosis, which suggested that miR155 was likely involved in the ischemic cerebral injury mediated by the mTOR signaling pathway. They proved that miR-155 combined with the 3'-untranslated region of Rheb mRNA and further inhibited the Rheb expression at the posttranscriptional level[26]. Guoping Xing et al. demonstrated that the expression of miR-155 increased in the cerebral tissues of MCAO rats with ischemia. On the contrary, the expression of Rheb and mTOR decreased at the protein level. Exogenous miR-155 inhibitors downregulated miR-155 expression but upregulated Rheb and mTOR expression, showing a protective effect in the injury process of IS. This protective role was characterized by a decrease in the infarct size and decreased apoptosis rate[27]. The results of the present study showed that the expression of miRNA155 increased in ischemia–reperfusion injury, which showed opposite changes with the ischemia–reperfusion injury extent, and this was

consistent with the results of the aforementioned studies[26, 27]. Therefore, therapeutic approaches that targeted miR-155 probably worked in IS [42].

The target of rapamycin (TOR) was first discovered in yeast using a mutant of a protein that caused resistance to the negative effects of rapamycin on growth [43]. Two homologs of TOR are present in yeast, known as TOR1 and TOR2. Interestingly, only one homolog of TOR was found in mammalian cells and was called mTOR, which played an essential role in the initiation of translation, transcription, organization of the cytoskeleton, and cell growth, proliferation, and survival [44–48]. The activity of mTOR was maintained at the degree of its normal function of equilibrium by combining several proteins with mTOR to form two complexes, named as mTORC1 and mTORC2[49], to realize the biological function of mTOR. Compared with mTORC2, mTORC1 has been found to be more sensitive to rapamycin [50]. Phosphorylation on its specific residues at the C-terminal of mTOR regulated its activity, including serine2448, the target of Akt and p70 ribosomal S6 kinase (p70S6K) [51–53], threonine2446, the target of AMP-activated protein kinase and p70S6K [51, 54], and serine2481, a nonsensitive rapamycin autocatalytic site of mTOR [55, 56]. Rheb is a small Guanosine Triphosphate enzyme(GTPase) regulating the survival, growth, and differentiation of cells by enhancing the signaling of the mTORC1 pathway[57]. Moreover, Rheb is the necessary protein in phosphorylating and activating mTOR[24]. Activated Rheb–GTP can directly combine with Raptor and further activate mTORC1 and regulate 4EBP1 to combine with mTORC1[58]. P70S6K and 4EBP1 are the two primary well-demonstrated downstream targets of mTORC1. Active mTORC1 can phosphorylate these two molecules to further activate p70S6K and inactivate 4EBP1. Activated p70S6K accelerates the biogenesis of mRNAs, translation of ribosomal proteins, and growth of cells [59, 60]. When mTORC1 is inhibited, nonphosphorylated 4EBP1 competitively binds with eukaryotic translation initiation factor 4G (eIF4G) to eIF4E. The combination of eIF4G with eIF4E is essential for initiating translation by interacting with the 5'-mRNA cap structure. The mTORC1 phosphorylates 4EBP1 and further causes the latter to dissociate from eIF4E, thus allowing eIF4G to combine with eIF4E and accelerate the initiation of translation [14, 61]. Several studies demonstrated that regulating mTOR activity had the potential of neuroprotection during IS. The application of estradiol to adult female ovariectomized rats before focal cerebral ischemia obviously reduced infarct volumes and apoptosis in the cerebral cortex and, at the same time, prevented the decline in the expression of phosphorylated mTOR and p70S6K induced by ischemia[62]. The downregulation of S6K1 accelerated injury in astrocytes induced by oxygen–glucose deprivation (OGD), an *in vitro* model of ischemia; on the contrary, the downregulation of S6K1 through adenoviral infection relieved cell injury[63]. Furthermore, the knockout of S6K increased the infarct volume and the mortality of mice with focal cerebral ischemia [63]. Moreover, the downregulated mTOR activity induced by rapamycin made cell survival tough and facilitated apoptotic injury in neural cells enduring OGD [64–66]. Erythropoietin has been demonstrated to defend microglia from OGD by promoting the mTOR activity and inhibiting the release of mitochondrial cytochrome c because mTOR inhibition through rapamycin silences the cell-protective function of erythropoietin [65]. Rapamycin can also enlarge the brain infarct size and increase the neurological deficit score in rats with focal cerebral ischemia, indicating that promoting mTOR activation may lead to lighter ischemic brain injury and better behavior recovery. Previous studies found

that Rheb mRNA levels were upregulated in IS, which was consistent with the results of the present study[67]. However, the protein level was downregulated in IS[27], indicating that the Rheb expression was regulated at the posttranscriptional levels and MiR-155 might directly act on Rheb mRNA. Although the protein and phosphorylation levels of the aforementioned molecules of the mTOR signaling pathway were not tested in this study, previous studies showed that the total protein levels of mTOR, S6K1, and 4EBP1 were upregulated while the phosphorylation levels were downregulated (please see the complementary data, unpublished).

Conclusions And Perspectives

In conclusion, the significance of this study was that it provided some specific proofs to predict the relationship between miR-155 and the Rheb/mTOR signaling pathway. The following hypothesis was made: in the ischemia–reperfusion process, the infarction area increases partly due to increased miR155 expression, leading to the inhibition of Rheb/mTOR signaling pathway and further activation of autophagy and apoptosis. MiR-155 may enhance ischemia–reperfusion injury by inhibiting the activation of the Rheb/mTOR signaling pathway, likely to act on Rheb mRNA at the posttranscriptional level and affect its translation. Considering there was no difference between the IP area and the ischemic core, intervention measures on miR-155 taken as soon as possible after the IS onset may be effective in decreasing the ischemia–reperfusion injury. However, this study had some limitations. It was not possible to surely explain that mRNA miR-155 acted on which target of Rheb; whether MiR-155 acted indirectly on mTOR, S6K1, and 4EBP1; and whether miR-155 accelerated the ischemic injury by macrophages. However, this study still provided an informative explanation for the lesion process of IS and might assist in developing early intervention strategy for this disease.

This indicated that the posttranslational regulation occurred rather than posttranscriptional regulation. The mechanism of the specific posttranslational regulation underlying cerebral ischemia and its correlation with MiR-155 level indirectly should be further investigated.

Moreover, the autophagy activity was upregulated probably due to the downregulation of the phosphorylation levels of mTOR, S6K1, and 4EBP1, as found in a previous study (please see the complementary data, unpublished). However, the mechanisms of autophagy underlying cerebral ischemia and whether such level of autophagy activity accelerated the death of neurons in cerebral ischemia should be further investigated. The time point at which autophagy is harmful is also important, in which autophagy inhibitors and activators should be administered.

In addition, this study deduced that some direct interactions existed between miR-155 and Rheb mRNA. Combined with the previous study results, it was inferred that autophagy might participate in the aforementioned process, which deserves further verification.

Materials And Methods

Animals

Thirty-six adult male Sprague–Dawley (SD) rats (Laboratory Animal Center of Xi'an Jiao Tong University) with an average age of 26 weeks and average weight of 250–280 g were used in this study. They were randomly divided into two groups (18 in each group): sham group (only artery was dissociated) and model group, both of which were further randomly divided into three subgroups (6 in each group) according to the post-surgery time (24-h group, 48-h group, and 72-h group). All rats were raised in a special pathogen-free animal house under standard conditions with a 12-h light–dark cycle, $60\% \pm 5\%$ humidity, and $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ ambient temperature for 3 days, during which enough water and pelleted food were supplied and all harmful stimulations were avoided.

Middle cerebral artery occlusion (MCAO)

The MCAO model of SD rats was constructed according to the instructions of previous reports of Zea-Longa[28]. Briefly, after adaptation for 3 days in the animal house with no food or drink for 12 h, the male SD rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate with a dose of 350 mg/kg and then fixed on the laboratory table. Afterward, a surgical midline incision was performed on the abdominal side of the neck, and then the right common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were exposed and isolated. ECA was ligated at the bifurcation of ICA and ECA, CCA was ligated at the near-heart end, a knot was made at the beginning of ICA, and a “v”-shape mouth was cut below the bifurcation of ICA and ECA. Soon afterward, a nylon suture (poly-L-lysine-coated monofilament nylon suture, 0.36 ± 0.02 mm diameter) was gently inserted into the right CCA lumen and then gently injected into the ICA till the beginning of the middle cerebral artery for approximately 1.8–2.0 cm when feeling a sense of resistance, implying that the blood flow of the middle cerebral artery was blocked. The previous knot at the beginning of ICA was turned into a firm knot to fix the suture, and then the skin of the neck incision was sewn up. After 90 min of blocking, the nylon sutures were gently removed from the ICA and reperfusion for 24, 48, or 72 h was performed. Once the aforementioned steps were successfully implemented, the neck incision was closed. An automatic homeothermic blanket control unit was used throughout the surgical procedure and postsurgery recovery to constantly monitor and maintain the body temperature of rats at 37°C . The rats in the sham group endured the same surgical operations except inserting the monofilament nylon suture.

3, 7-Triphenyltetrazolium chloride staining for evaluating cerebral infarction in animal models

After ischemia for 90 min, the model rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and were sacrificed respectively at 24, 48, and 72 h post-reperfusion. The rat brains were rapidly and gently removed and frozen at -20°C for 20 min. Frozen brain tissues were dissected and sliced into 2-mm coronal sections. The slices were stained with 2% 2, 3, 7-triphenyltetrazolium chloride

(TTC, Sigma, United States of America, USA) at 37°C for 15 min in the dark; then, they were fixed in 10% formaldehyde buffer for 30 min. Next, they were placed on a medical blue sheet and photographed using a digital camera. The ImageJ 1.46R software (NIH, USA) was used to determine the cerebral infarction area, model hemisphere volume, and contralateral hemisphere volume so as to reduce the influence of brain edema on the infarction volume. The adjusted formula was used to calculate the infarction volume, which was expressed as a ratio: percentage of corrected infarct volume = $\{[\text{Total lesion volume} - (\text{ipsilateral hemisphere volume} - \text{contralateral hemisphere volume})] / \text{contralateral hemisphere volume}\} \times 100\%$.

RNA isolation

After reperfusion for 24, 48, and 72 h, the rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and were sacrificed respectively, and the brain tissues were rapidly taken out on the ice. Afterward, the ischemic core, boundary zone, and relative contralateral brain tissues were divided and weighed separately. The corresponding matched cerebral tissues were taken out in the sham groups. As suggested, 1 mL of TRIzol reagent (Invitrogen, USA) was added to every 100 mg cerebral tissue and homogenized rapidly. Total RNA was isolated from tissues following the manufacturer's protocol of TRIzol reagent. The NanoDrop ND-1000 (Thermo Fisher Scientific, USA) ultraviolet spectrophotometer was used to determine the concentration and the purity of the RNA, and the agarose gel electrophoresis method was used to test the integrity of the RNA.

Reverse transcriptase–polymerase chain reaction

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was used to detect the miR-155 level in different brain tissues. Total RNA that proved to be well qualified was reverse transcribed into cDNA of miR-155 and U6 snRNA following the manufacturer's protocol of TaqMan MicroRNA Reverse Transcription Kit, and the cDNA was stored at -20°C for further use. Then, the qRT-PCR was performed to detect the miR-155 and U6 levels following the manufacturer's protocols of TaqMan MicroRNA assays, TaqMan Universal PCR Master Mix II, and the Uracil N-Glycosylase (UNG) Kit (Applied Biosystems, USA). Three identical panels were designed for each reaction. Also, U6 was chosen as the internal reference of miR-155 expression. qRT-PCR of the SYBR green method was used to detect the mRNA levels of Rheb, mTOR, S6kb1, and 4Ebp1 in different brain tissues. Briefly, RT-PCR was carried out using the PrimeScript RT Reagent Kit with gDNA Eraser and SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, China) with Bio-Rad iQ5 Real-Time PCR System (Bio-Rad, USA). The target gene expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the $2^{-\Delta\Delta CT}$ method was used to quantify the target gene expression.

Statistical analysis

Statistical analyses were conducted using the SPSS 21.0 software (IL, USA), and the data were expressed as mean $\pm$ standard deviation. Differences in continuous variables between two groups were investigated using the two-tailed Student *t* test in which statistical significance was determined when the *P* value of the corresponding statistical tests was less than 0.05.

Abbreviations

Rheb: Ras-homolog enriched in brain

TOR: target of rapamycin

TOR1: target of rapamycin 1

TOR2: target of rapamycin 2

mTOR: mammalian target of rapamycin

mTORC1: mTOR complexes 1

mTORC2: mTOR complexes 2

MCAO/R: middle cerebral artery occlusion/ reperfusion model

IP: ischemic penumbra

S6kb1: Ribosomal S6 kinase 1

ATP: adenosine triphosphate

eIF4E: eukaryotic initiation factor 4E

4EBP1: eIF4E binding protein 1

FDA: Food and Drug Administration

MiRNA155: Micro Ribonucleic acid 155

IS: ischemic stroke

RPS6KB2: Ribosomal Protein S6 Kinase polypeptide 2,

RICTOR: Rapamycin insensitive companion of mTOR

SD: Sprague–Dawley

TTC:2,3,5,7-Triphenyltetrazolium chloride

CCA: common carotid artery

ICA: internal carotid artery

ECA: external carotid artery

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

qRT-PCR: quantitative reverse transcriptase–polymerase chain reaction

p70S6K: p70 ribosomal S6 kinase

GTPase: Guanosine Triphosphate enzyme

OGD: oxygen–glucose deprivation

ANOVA: analysis of variance

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the Xi'an Central Hospital, Medical College of Xi'an Jiao Tong University, Xi'an Shaanxi, China, under protocol LW-2021-002, for the use of animal specimens. Animal raising and experimental protocols followed the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals. We confirmed that the study is reported in accordance with ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Yu-E Yan, Wen-Zhen Shi, Bin Li and Ye Tian conceived and designed the study. Jing Xiong provided the supplementary data. Xu-Rong, Zhu and Fang He performed the research and analyzed the data. Zhi-Qin Liu, Nai-Bin Gu, Xiao-Tao Jia, Ge-Min Zhu and Jing-Jing Du provided help and advice on the model establishment and data analysis. Zheng-li Di and Hua Guo instructed and supervised the implementation of the whole study. Yu-E Yan wrote the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

TTC staining results of MCAO/R model rats' brain tissues at different re-perfusion time. (A) Representative photographs of TTC-stained brain slices of different levels in different groups (sham, 24 h, 48 h, and 72h after reperfusion). The error bar represents 1.0 cm. (B) Relationship between infarction volume ratio and the reperfusion time is shown with a column chart. Data were depicted as mean $\pm$ standard deviation ($n = 4$), and a two-tailed t test was used to compare the infarction volume ratio of the model group and time-matched sham group. The P values are depicted with different signs (*, #, and &). All of them showed statistical significance. * $P < 0.05$, time-matched sham group versus the model group; # $P < 0.05$, 24-h model group versus the 48-h model group; and & $P < 0.05$, 48-h model group versus the 72-h model group.

Figure 2

Figure 2

Upregulation of miR-155 levels in brain tissues of model rats. The quantified levels of miR-155 in the brain tissues in both the boundary zone and the ischemia core of different reperfusion groups are demonstrated with a column chart (A) and a line chart (B), with U6 as the inner reference. Statistically significant differences compared with that of the time- and area-matched sham group are marked on the top; data of both boundary zone (* $P < 0.05$) and ischemia core (# $P < 0.05$) are presented. All the data are from four independent assays and presented as mean $\pm$ standard deviation ($n = 4$).

Figure 3

Figure 3

Upregulation of Rheb mRNA levels in the brain tissue of model rats. The quantified mRNA level of Rheb in the brain tissues of different groups with GAPDH as the reference is presented with a column chart (A) and a line chart (B). Statistically significant differences compared with that of the time- and area-matched sham group are marked on the top; data of both boundary zone (* $P < 0.05$) and ischemia core (# $P < 0.05$) are presented. All data are from four independent assays and presented as mean $\pm$ standard deviation ($n = 4$).

Figure 4

Figure 4

Upregulation of mTOR mRNA in the brain tissue of model rat. The quantified mRNA levels of mTOR in the brain tissues of different groups with GAPDH as the reference are demonstrated with a column chart (A) and a line chart (B). Statistically significant differences compared with that of the time- and area-matched sham group are marked on the top; data of both boundary zone (*,& $P < 0.05$) and ischemia core ($\#P < 0.05$) are presented. All data are from four independent assays and presented as mean $\pm$ standard deviation ($n = 4$).

Figure 5

Figure 5

Upregulation of S6kb1 mRNA in the brain tissue of model rat. The quantified mRNA levels of S6kb1 in the brain tissues of different groups with GAPDH as the reference are demonstrated with a column chart (A) and a line chart (B). Statistically significant differences compared with that of the time- and area-matched sham group are marked on the top; data of both boundary zone (*P < 0.05) and ischemia core (#P < 0.05) are presented. All data are from four independent assays and presented as mean ± standard deviation (n = 4).

Figure 6

Figure 6

Upregulation of 4Ebp1 mRNA in the brain tissue of model rat. The quantified mRNA levels of 4Ebp1 in the brain tissues of different groups with GAPDH as the reference are demonstrated with a column chart (A) and a line chart (B). Statistical differences compared with that of the time- and area-matched sham group are marked on the top; data of both boundary zone (*P < 0.05) and ischemia core (#P < 0.05) are presented. All data are from four independent assays and presented as mean ± standard deviation (n = 4).

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