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EFFECTS OF MTOR ON NEUROLOGICAL DEFICITS AFTER TRANSIENT GLOBAL ISCHEMIA

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Abstract

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase and activation of its signal pathway plays an important role in regulating protein growth and synthesis as well as cell proliferation and survival. In the present study, we examined the contribution of mTOR and its downstream products to brain injuries and neurological deficiencies after cardiac arrest (CA) induced-transient global ischemia. CA was induced by asphyxia followed by cardiopulmonary resuscitation (CPR) in rats. Our results showed that expression of p-mTOR, mTOR-mediated phosphorylation of 4E–binding protein 4 (4E-BP1) and p70 ribosomal S6 protein kinase 1 (S6K1) pathways were amplified in CA rats compared to their controls. Blocking mTOR using rapamycin attenuated upregulation of pro-inflammatory cytokines (namely IL-1β, IL-6 and TNF-α), and Caspase-3, indicating cell apoptosis and also promoting the levels of vascular endothelial growth factor (VEGF) and its subtype receptor VEGFR-2 in the hippocampus. Moreover, the effects of rapamycin were linked to improvement of neurological deficits and increased brain water content observed in CA rats. In conclusion, activation of mTOR signal is engaged in pathophysiological process during CA-induced transient global ischemia and blocking mTOR pathway plays a beneficial role in regulating injured neuronal tissues and neurological deficits via PIC, apoptotic Caspase-3 and VEGF mechanisms. Targeting one or more of these specific mTOR pathways and its downstream signaling molecules may present new opportunities for neural dysfunction and vulnerability related to transient global ischemia.

Keywords

• cardiac arrest • cardiopulmonary resuscitation • hippocampus • mTOR • rapamycin • cytokines • Caspase-3

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Introduction

When cardiac arrest (CA) occurs, blood flow and oxygen delivery are abruptly halted and these responses lead to systemic ischemic injury in various organs, including the brain [1]. Although cardiopulmonary resuscitation (CPR) is applied, inadequate blood flow and tissue oxygen delivery still persist due to myocardial dysfunction, hemodynamic instability and microvascular dysfunction. In response to ischemic and hypoxic insults, neuroprotective mechanisms are engaged in the ischemic brain by improving the permeability of blood brain barrier, reducing brain edema formation and promoting the recovery of brain injuries [2]. Thus, it is important to study signal pathways and further determine biological agents involved in exerting neuroprotective effects and attenuating damage evoked by global cerebral ischemia.

Previous studies have demonstrated that hypoxia inducible factor-1 (HIF-1) subtype HIF-1α is expressed in brain tissues, including cerebral cortex and hippocampus, and engages in neuronal apoptosis after induction of global ischemia [3-6]. In particular, our recent study using this model has further demonstrated that CA increases IL-1β, IL-6 and TNF-α and upregulates their receptors IL-1R, IL-6R and TNFR1 in the hippocampus [7]. Systemic administration of ML228, an activator of HIF-1α [8], attenuates these exaggerated pro-inflammatory cytokines (PIC) signal pathways [7], and as a result HIF-1α activation and/or stabilization, improves neuronal apoptosis and neurological deficits induced by CA [9].

Vascular endothelial growth factor (VEGF) is an important signalling protein, engaged in both vasculogenesis and angiogenesis, which leads to restoring the blood supply to tissues [10]. It has been shown that VEGF stimulates endothelial cell mitogenesis and cell migration, but also enhances microvascular permeability [10]. Moreover, it has been reported that VEGF is up-regulated after CA-evoked cerebral ischemia and this likely improves the permeability of blood-brain barrier, reduces brain edema formation and promotes the recovery of brain injuries [2, 11].

Mammalian target of rapamycin (mTOR), including complex 1 (mTORC1) and complex 2 (mTORC2), is a serine threonine protein kinase. Specifically, mTORC1 is more sensitive to rapamycin and its activation promotes the phosphorylation of downstream effectors, such as p70 ribosomal S6 protein kinase (p70S6K) which further governs mRNA translation [12]. The mTORC1 is well known for its critical roles in the regulation of protein synthesis and growth, and it has been reported to be involved in ischemic injuries after intracerebral hemorrhage. Nonetheless, the precise signal

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pathways for mTOR in the process of CA-evoked cerebral ischemia are largely unknown.

Accordingly, in the present study, we examined responsiveness of mTOR signal pathway in the brain tissues of control and CA rats and further determined whether PIC, such as IL-1β, IL-6 and TNF-α, and Caspase-3, as an indicator of neuronal apoptosis, are engaged in the effects of mTOR signal on neurological deficits evoked by CA. We hypothesized that CA increases p-mTOR protein expression, mTOR-mediated phosphorylation of 4E–binding protein 4 (p-4E-BP1) and p70 ribosomal S6 protein kinase 1 (p-S6K1) pathways. We further hypothesized that blocking mTOR attenuates upregulation of PICs and Caspase-3 induced by CA, and promotes VEGF and its subtype receptor VEGFR-2, thereby improving neurological deficits and amplified brain water content induced by CA.

Materials and methods

Experimental Animals

All the animal procedures were approved by the Institutional Animal Care & Use Committee of Jilin University, which were in compliance with the guidelines of the International Association for the Study of Pain and the Guideline for the Care and Use of laboratory Animals of the U.S. National Health Institute. Male Sprague–Dawley rats (200–300g) were used in our experiments.

Transient global ischemia induced by CA

The ischemia was produced in the CA model induced by asphyxia. Briefly, rats were anesthetized with an isoflurane-oxygen mixture (2-5% isoflurane in 100% oxygen). An endotracheal tube was first inserted and attached to a ventilator. The ventral tail artery was cannulated to monitor systemic arterial pressure. The right jugular vein was cannulated for a continuous infusion of saline (at a rate of 0.1 ml/hour) to maintain baseline blood pressure and fluid balance. Body temperature was continuously monitored and maintained at 37°C with a heating pad and external heating lamps. Asphyxia was induced by stopping mechanical ventilation and clamping the tracheal tubes at the end of expiration. Resuscitation efforts began 6 min following induction of CA. For this purpose, rats were orotracheally intubated for mechanical ventilation accompanied by chest compression delivered by a mechanical compressor at a rate of 200/min for 5 min. Once spontaneous heartbeat returned, epinephrine (2 μg) was administered to achieve a mean arterial blood pressure of >80 mmHg. Ventilation was adjusted for animals to regain spontaneous respiration and achieve normoxia. The animals that survived from this procedure were used for further interventions. For pain relief those rats received analgesic buprenorphine (0.02 mg/kg) subcutaneously immediately and every 6 hours for a 72-hour period after the asphyxial procedures. In the sham operated animals, the same surgical procedures were performed without cardiac arrest and resuscitation.

Study interventions and animal groups

The rats were divided randomly. Group 1: the rats received the same surgical procedures and endotracheal intubation was performed with no asphyxia and CPR. Group 2: CA and CPR were performed and 0.5 ml of saline (i.p., every 12 hours for 3 days) was given after CA. Group 3: CA and CPR were carried out and 500 μg/kg body weight of rapamycin (i.p., every 12 hours for 3 days) was injected after CA. At the end of each experiment, the rats were sacrificed and then the brains were taken out for biochemical measurements. Note that the hippocampus CA1 region was used in this study.

Neurological examination

The modified method of Neurological Severity Score (mNSS) was used to examine neurological functions in this study [13]. Note that mNSS was generally used to assess a combination of motor, sensory, and balance functions. Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18). In case of mNSS score of rats > 0 before CA, they were considered abnormal and thus were excluded from the experiment. Experiments were performed in a blind manner.

Brain edema (brain water content) was determined 72 hours after CA. The brain slices (2 mm thick) of the hemispheres and cerebellum were cut. The whole brain water content was calculated from all slices [14, 15]. The brain slices were weighed to obtain the wet weight immediately and dried in an oven at 100ºC for 24 hours to obtain the dry weight. The cerebellum was used as the internal control. The water content was expressed as the following formula:

[(wet weight) – (dry weight)]/(wet weight) × 100%.

ELISA measurements

All the tissues from individual rats were sampled for the analysis. In brief, the hippocampus CA1 region of the rats was removed under an anatomical microscopy. Total protein was then extracted by homogenizing the hippocampus sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay (BCA) reagent kit.

The levels of PICs were examined using an ELISA assay kit (Promega Co. Madison, WI) according to the provided description and modification. Briefly, polystyrene 96-well microtitel immunoplates were coated with affinity-purified rabbit anti-IL-1β, anti-IL-6 and anti-TNF-α antibodies. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation, plates were washed. Then, the diluted samples and these PIC standard solutions were distributed in each plate. The plates were washed and incubated with anti- IL-1β, IL-6 and TNF-α galactosidase. Then, the plates were washed and incubated with substrate solution. After incubation, the optical density was measured using an ELISA reader (at 575 nm wavelength). This method was also employed to examine the levels of VEGF according to the instructions of the manufacturer (Promega Corp. Madison, WI).

Western blot analysis

The brain tissues were homogenized and centrifuged to measure total protein. After being denatured by heating at 95°C in an SDS sample buffer, the supernatant samples were loaded onto gels and then electrically transferred to a

polyvinylidene fluoride membrane. The protein expression of mTOR, S6K1, 4E-BP1 and their respective phosphorylated forms, i.e. p-mTOR, p-S6K1, p-4E-BP1 as well as VEGFR-2 and a cleaved form of Caspase-3, were determined using a standard Western blot analysis. In brief, membranes were incubated with rabbit anti-p-mTOR/p-S6K1/p-4E-BP1 antibodies; rabbit anti-mTOR/S6K1/4E-BP1 antibodies; and rabbit anti-VEGFR-2 and anti-Caspase-3 (1:500, obtained from Abcam Co.). After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-rabbit secondary antibody (1:250) and visualized for immunoreactivity. The membrane was also processed to detect β-actin for equal loading. The bands recognised by the primary antibody were visualised by exposure of the membrane onto an X-ray film. The film was then scanned and the optical densities of protein bands were analysed using the Scion image software (obtained from the NIH), and values for densities of immunoreactive bands/β-actin from the same lane were determined. Each of the values was then normalised to a control sample [7, 9].

Statistical Analysis

A two-way repeated-measure analysis of variance was used to analyse all the data in this report as a multiple set of data were compared between two groups or among three groups. Values were presented as means ± standard deviation. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 15.0 (SPSS, USA).

Results

Expression of mTOR signal pathway

Figure 1 demonstrates the protein expression of p-mTOR, p-S6K1 and p-4E-BP1 as well as total protein levels of mTOR, S6K1 and 4E-BP1 in control and CA rats. CA significantly increased p-mTOR, mTOR-mediated p-S6K1 and p-4E-BP1 protein levels in the CA1 region compared to control rats ($P < 0.05$ *vs.* control rats; n=6-8 in each group). However, total protein levels of mTOR, S6K1 and 4E-BP1 were not significantly altered in CA rats ($P > 0.05$ *vs.* control rats; n=6-8 in each group).

Effects of blocking mTOR on PICs, VEGF and VEGFR-2, and Caspase-3

Figure 2A shows that IL-1β, IL-6 and TNF-α were significantly elevated in the CA1 subfield of the hippocampus after induction of CA ($P < 0.05$ *vs.* control rats; control rats =10 and CA rats =12) compared to control rats. Inhibition of mTOR, by administration of rapamycin, significantly decreased the amplification of IL-1β, IL-6 and TNF-α in CA rats ($P < 0.05$ *vs.* CA rats; n=15 in CA rats with rapamycin).

Figure 2B illustrates that induction of CA significantly increased the levels of VEGF in the CA1 region compared to control rats ($P < 0.05$ *vs.* control rats; control rats =10 and CA rats =12). Figure 2C further shows that CA amplified the protein expression of VEGFR-2 and Caspase-3 in the CA1 of rats ($P < 0.05$ *vs.* control rats; n=6 in each group). As rapamycin was given in CA rats to block mTOR signal pathway, VEGF and VEGFR-2 expression resulted enhanced and Caspase-3 expression attenuated.

Neurological function and brain edema

The survival rate resulted increased during 72 hours when CA rats received rapamycin treatment compared to CA rat without rapamycin. The survival rate of 72 hours was 100% (10/10 rats) in sham control rats; 63% (12/20 rats) in CA rats; 88% (15/17 rats) in CA rats injected with rapamycin. Data obtained from those survival rats were included for the analysis in this report.

Figure 3A and demonstrate that CA led to mNSS score and water content increase ($P< 0.05$ *vs.* control rats; n=10 in control and n=12 in CA group). Also, the figures show

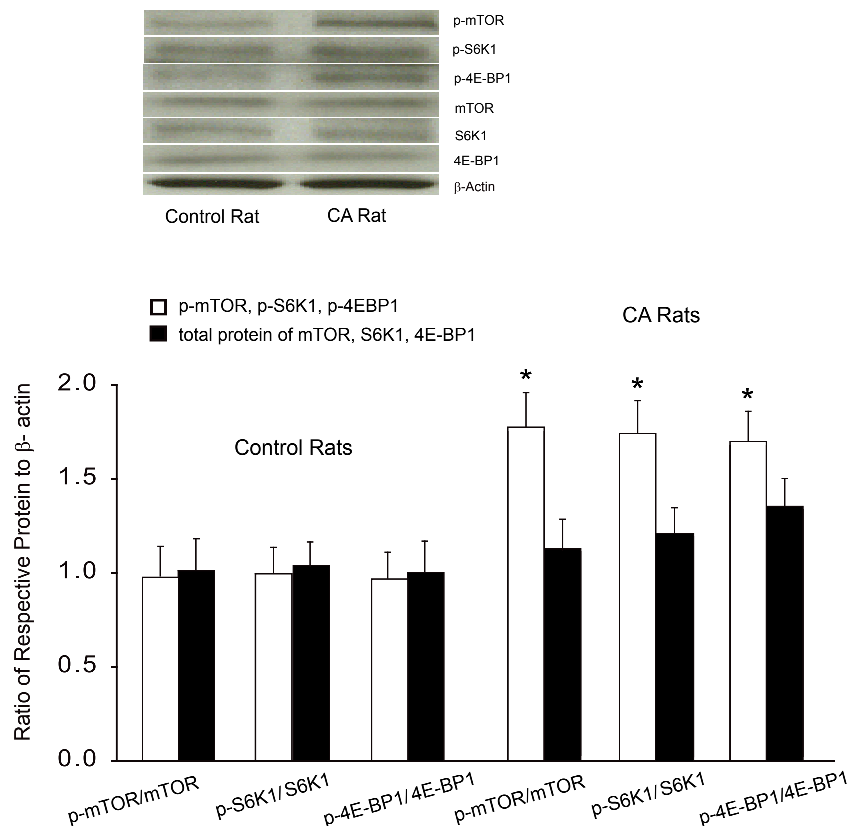

Figure 1. Expression of mTOR and its downstream signal. Typical bands (top panels) and averaged data (bottom panel): Showing p-mTOR, p-S6K1 and p-4E-BP1 in the CA1 region of control rats and ischemic rats evoked by CA. Also, total protein of mTOR, S6K1 and 4E-BP1 was shown. Cerebral ischemia failed to significantly increase the total protein levels of mTOR, S6K1 and 4E-BP1, but significantly elevated p-mTOR, p-S6K1 and p-4E-BP1. *P<0.05 *vs.* respective protein in control rats. n=6-8 in each group.

that administration of rapamycin significantly attenuated the increased mNSS and amplified water content of the hippocampus tissues in CA animals (*P*< 0.05, CA rats *vs*. CA rats with rapamycin; n=15 in CA rats with rapamycin).

Discussion

There are two distinct mTOR forms of protein complexes, mTORC1 and mTORC2. In general, mTORC1 is composed of raptor, mLST8 and mTOR, and is known to gate translation of most proteins by phosphorylation of specific downstream effectors including, p70 ribosomal S6 protein kinase (p70 S6Ks) and 4E-BPs [12]. mTOR, S6K1 and 4E-BP1 are expressed in the mammalian central nervous system [16-18]. The mTORC1 plays a critical role in the regulation of protein synthesis and growth and further compelling evidence supports the notion that mTOR plays an important role in the modulation of long-term neuronal plasticity [16, 19]. Specifically, data from this study indicate the contribution of mTOR and its downstream effectors to neuronal injury in the process of CA-induced transient global ischemia.

Prior studies have demonstrated that HIF-1α is expressed in the brain tissues including the cerebral cortex and hippocampus and engaged in neuronal apoptosis after induction of global ischemia [3-6]. In particular, a recent study using this model has further demonstrated that CA increases IL-1β, IL-6 and TNF-α and upregulates their receptors IL-1R, IL-6R and TNFR1 in the hippocampus [7]. Systemic activation of HIF-1α attenuates these exaggerated PIC signal pathways [7], and as a result neuronal apoptosis and neurological deficits induced by CA are attenuated [9]. In the present study, we administered the mTOR inhibitor rapamycin and examined its effects on the upregulation of PICs in the CA1 region of CA rats. This suggested that PIC mechanisms are engaged in the protective role played by blocking mTOR signal in the pathophysiological process of transient global ischemia, linked to neurological deficits observed in CA rats.

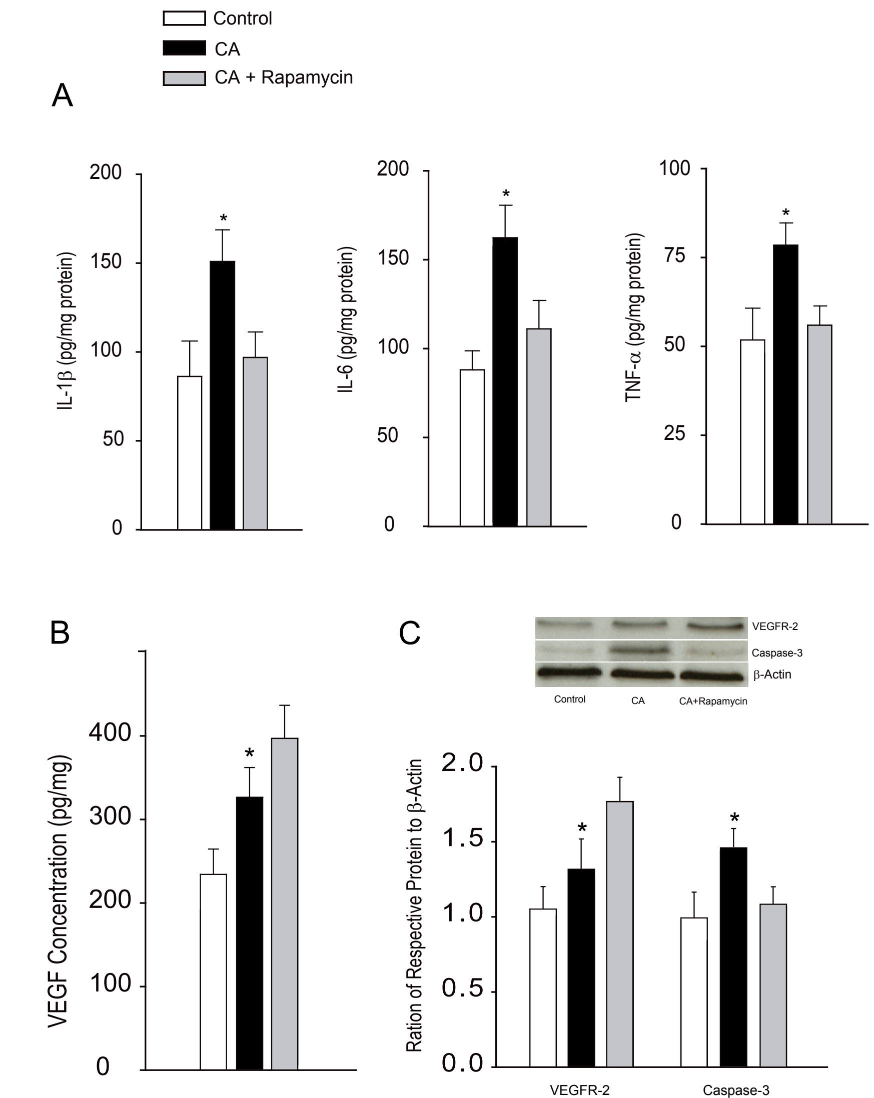

Figure 2. Effects of blocking mTOR on PICs, VEGF, VEGFR-2, and Caspase-3. (A): The levels of IL-1β, IL-6 and TNF-α in the hippocampus CA1 region. Cerebral ischemia evoked by CA amplified the levels of these PICs. Inhibition of mTOR signal, using rapamycin, decreased the augmented IL-1β, IL-6 and TNF-α in the CA1 region of CA rats. **P* < 0.05, CA rats (n=12) *vs.* control rats (n=10) and CA rats with rapamycin (n=15). (B): CA increased the levels of VEGF in the CA1 region of rats and VEGF was amplified by rapamycin. **P* < 0.05 *vs.* control rats CA rats with rapamycin. N=10 in control; n=12 in CA rats and n=15 in CA rats with rapamycin. (C): CA increased the protein expression of VEGFR-2 and Caspase-3 in the hippocampus of rats; administration of rapamycin amplified upregulation of VEGFR-2 and attenuated Caspase-3. **P* < 0.05 *vs.* control rats and CA rats with rapamycin. n=6 in each group.

VEGF is an important signalling protein playing a role in regulating the neovascularisation as a downstream product of HIF-1α, which contributes to VEGF formation due to ischemic or hypoxic conditions [10]. Also, VEGF and its subtype receptor VEGFR-2 play an important role in pathologic angiogenesis [20, 21]. Amplified HIF-1α, VEGF and VEGFR-2 have been found in the brain tissues after induction of CA and promotion of VEGF and VEGFR-2 by stabilisation of HIF-1α can improve neurological deficits and increased brain water content induced by ICH [22]. Nevertheless, results of our current study provide evidence that blocking of mTOR signal plays a beneficial role in modulating brain injuries observed in CA rats via VEGF mechanisms.

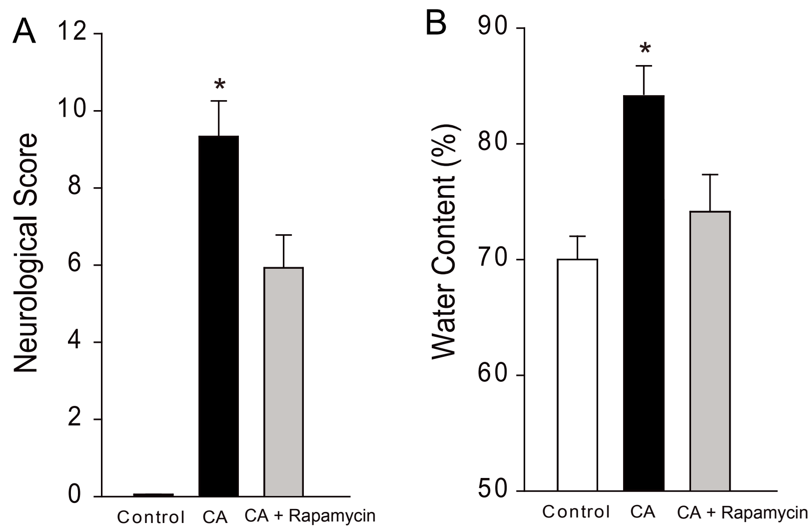

Figure 3. Effects of blocking mTOR on mNSS and water content. (A) and (B): CA increased the mNSS and water content of brain tissues compared to control animals. As rapamycin was given the increased mNSS and water content were attenuated in CA rats. $^*P < 0.05$ *vs.* control rats and CA rats with rapamycin. N=10 in control; n=12 in CA rats and n=15 in CA rats with rapamycin.

Of note, VEGF regulates vascular development, angiogenesis and lymphangiogenesis by binding to a number of VEGFRs [23, 24]. There are three main subtypes of VEGFR, numbered 1, 2 and 3. The function of VEGFR-1 is less defined, although, it is required for the recruitment of haematopoietic stem cells and the migration of monocytes and macrophages [23, 24]. VEGFR-2 mediates almost all of the known cellular responses to VEGF and it is critical for vascular endothelial cell development and regulates vascular endothelial functions [23]. VEGFR-3 regulates lymphatic endothelial cell function and mediates lymphangiogenesis in response to VEGF [23, 24]. Considerable evidence shows that VEGFR-2 is specific intracellular signal cascades leading to proliferation, migration, survival and increased permeability, each of which contributes to the angiogenic response [23]. Accordingly, in the present study, we examined the involvement of VEGF subtype receptor VEGFR-2 in mTOR signal pathway in the process of neurological deficits after CA.

Caspases, a family of thiol proteases, regulate the apoptotic cascade and induction of CA activates neuronal caspases. In the death-signalling cascade, it is crucial that pro-caspase-3 is processed to its active form Caspase-3, and it has been reported that Caspase-3 is a principal target engaged in the reactive oxygen species-mediated apoptosis in human endothelial cells [25]. Thus, in the current study, we also determined the levels of cleaved Caspase-3 in the hippocampus tissues, as an indicator of cellular apoptosis, and we found that CA increases Caspase-3 in CA animals. This is consistent with the results observed in a previous study [9]. Moreover, inhibition of mTOR decreases the amplified expression of Caspase-3 evoked by CA.

A rat model of CA-induced cerebral ischemia has been employed to determine the mechanisms responsible for neurological damage. This rat model was used in the current study showing that expression of p-mTOR and its downstream pathways, namely p-S6K1 and p-4E-BP1, are upregulated by CA. We further examined the effects of rapamycin on a number of critical molecular mediators involved in the pathophysiological process of CA, including PICs, VEGF, VEGFR-2 and Caspase-3. We observed that rapamycin attenuates amplification of IL-1β, IL-6, TNF-α and Caspase-3 induced by CA. In contrast, rapamycin enhances VEGF levels and VEGFR-2 expression in CA animals. Consistent with these results, our data further showed that damaged neurological scores and increased water content caused by CA are significantly improved by rapamycin. Overall, we suggest that inhibition of mTOR signal pathway is likely to play a beneficial role in regulating neurological deficits induced by CA via PICs and VEGF mechanisms.

In conclusion, activation mTOR signal is involved in pathophysiological process during CA-evoked transient global ischemia and blocking mTOR signal pathway plays a role in improving deteriorated neuronal tissues and neurological deficits via PIC, VEGF and apoptotic Caspase-3 mechanisms. Specifically, results of this study have pharmacological implications to target one or more mTOR and its downstream signalling molecules for neurological dysfunction and vulnerability related to CA.

Conflict of interests:

None

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