RNA interference-mediated downregulation of Beclin1 attenuates cerebral ischemic injury in rats

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Aim: To test the role of the Beclin 1-dependent autophagy pathway in brain damage during cerebral ischemia.

Methods: Focal cerebral ischemia was established in rats using a middle cerebral artery occlusion (MCAO) model. A lentiviral vector-associated RNA interference (RNAi) system was stereotaxically injected into the ipsilateral lateral ventricle to reduce Beclin1 expression. We measured the ipsilateral infarct volume, autophagosome formation, neurogenesis and apoptosis, all of which could be modulated by Beclin1 RNAi.

Results: On the 14th day after MCAO, Beclin1 downregulation by RNAi increased the population of neural progenitor cells (BrdU+-DCX+), newborn immature cells (BrdU+-Tuj-1+) and mature neurons (BrdU+-MAP-2+), and reduced the apoptosis of immature neurons (caspase-3+-DCX+ and caspase-3+-Tuj-1+) surrounding the ischemic core of the ipsilateral hemisphere. Furthermore, RNAi-mediated downregulation of Beclin1 decreased infarct volume and inhibited histological injury and neurological deficits.

Conclusion: RNAi-mediated downregulation of Beclin1 improves outcomes after transient MCAO.

Keywords: autophagy; beclin1; apoptosis; stroke; RNA interference

Introduction
Apoptosis and necrosis are important modes of neuronal death induced by cerebral ischemia, but in recent years a third method of death, autophagy, has been studied[1–3]. Autophagy, which involves the formation of autophagosomes and autophagolysosomes, has been studied extensively as a caspase-independent cell death pathway and has been associated with ischemia and reperfusion in the heart and brain[4–7]. Autophagy appears to occur after hypoxia-ischemia as an important mechanism of neuronal self-destruction, as well as after transient global ischemia and adult focal ischemia[6–10]. In addition, autophagy has also been recognized as a cell survival mechanism. It may protect neurons by eliminating damaged structures and delivering them to lysosomes for degradation[11, 12]. Hence, whether autophagy is a friend or foe during cerebral ischemia/reperfusion remains to be seen. We therefore used a rat middle cerebral artery occlusion (MCAO) model to induce transient focal brain ischemia in the present study.

Beclin1, the homologue of the yeast autophagy gene 6 (Atg6), was first dissociated from the inhibitory Beclin1-Bcl-2 complex and was suggested as a potential link between autophagy and apoptosis[13]. Beclin1-deficient mice show a pronounced loss of autophagic vacuole formation[14–16]. In mammals, Beclin1 downregulation by RNA interference (RNAi) blocked cell death induced by autophagy[17, 18]. Furthermore, induction of autophagy and cardiac injury during the reperfusion phase were significantly attenuated in Beclin1−/− mice[5]. As a rapamycin-independent autophagy mechanism[12], the role of the Beclin1-induced autophagy in brain ischemia needs more elucidation. Therefore, in the present study, lentiviral vectors encoding Beclin1 short hairpin RNA (LV-shBeclin1) were stereotaxically delivered into the ipsilateral lateral ventricle to inhibit ischemia-induced Beclin1 expression.

As previously reported, an increase in neurogenesis following ischemia has been considered to be necessary for functional recovery after stroke[19, 20]. Beclin1-dependent autophagy has been associated with neurogenesis in several models[21, 22]. These findings supporting the existence of a complex interplay between autophagy, cell proliferation and cell death during neural development[22]. However, it remains unknown whether autophagy has any influence on ischemia-induced neurogenesis. The present study addresses the role of Beclin1-dependent autophagy during the period of neurogenesis after transient MCAO.
Here, we demonstrate that RNAi-mediated downregulation of Beclin1 was protective following transient MCAO in vivo. Further, the present study is the first, to our knowledge, to examine the potential link between Beclin1-dependent autophagy, apoptosis, and neurogenesis in response to cerebral ischemia/reperfusion.

Materials and methods

Animals

Adult male Sprague-Dawley rats (250–270 g), provided by the Department of Animal, Health Science Center of Peking University, were housed in a laboratory animal room and maintained at 25±1 °C with 65%±5% humidity on a 12-h light/dark cycle (lights on from 07:30 to 19:30) for at least 1 week before the start of the experiments. Animals were given food and water ad libitum. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Xiyuan Hospital, China Academy of Chinese Medical Sciences.

Lentiviral vectors and shRNA injection

The design of the shRNA followed published guidelines[23]. Lentiviral vectors pGCL-LTR-RRE-U6 shRNA-CMV Luciferase-WRE-LTR (LV-shBeclin1) were generated. The shBeclin1 sense oligonucleotide sequence 5’-GGATCCCCACTCACAGTCCATTACCTATTTCAAGAGATAATG-GAGCTGTGATTITTGTTGATGCTT-3’ and the shBeclin1 antisense oligonucleotide sequence 5’-AAGCTATCCAAAATCTCAGCCTCCATTACCTATCTTGAATGTTAATGGAGCTGTGATTITTGTTGATGCTT-3’ were annealed and cloned into a pGC/U6 plasmid. One unrelated sequence (TTCTCCGAACGTGTCACGT) that shows no significant homology to any known rat gene (analyzed using a BLAST search) was used as an LV-control. Seven days before MCAO, 1.5 µL of concentrated viral solution was stereotaxically delivered into the ipsilateral lateral ventricle (coordinates from bregma: AP-0.8 mm, ML 1.4 mm, DV-3.6 mm from the pial surface).

Transient MCAO

Rats with physiological variables within normal ranges were subjected to transient focal cerebral ischemia induced by right MCAO as previously described[24]. In brief, rats were anesthetized with 3.5% chloral hydrate (350 mg/kg, ip) and were placed in a supine position. The body temperature of the rats was maintained at 37±0.5 °C during the surgical procedure. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were surgically exposed. The CCA was ligated distally and the ECA was ligated proximally to the bifurcation of the ICA and the ECA. A 3–0 silk suture was tied loosely around the ICA, and a microvascular clip was placed distally across the ICA. A 4–0 nylon monofilament suture with a rounded tip was inserted into the CCA through the ICA and gently advanced for a distance of 20 mm from the common carotid artery bifurcation to block the origin of the middle cerebral artery (MCA). The suture around the ICA was tightened, and the microvascular clip was removed. Mean arterial blood pressure, heart rate, and arterial blood gases were analyzed during the surgical procedure. After 60 min of MCAO, the suture was pulled back until the tip reached the suture around the ICA to restore blood flow (reperfusion). Regional cerebral blood flow (rCBF) was measured during the focal ischemia surgery. Laser-Doppler flowmetry (Periflux, Perimed) was used to monitor rCBF. Recordings were made bilaterally from each animal for at least 10 min before, during, and within 30 min after reperfusion. After the wound had been closed, the animals were allowed to recover from anesthesia before they were returned to their home cages.

Luciferase assay

Luciferase activity was quantified by a luciferase assay system (Promega, Madison, WI) according to the manufacturer’s protocol. The results were reported as luciferase activity/total protein.

Tissue preparation

Rats were sacrificed 24 h and 14 days after MCAO with an overdose of 3.5% chloral hydrate and were transcardially perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). Brains were removed, postfixed overnight, and equilibrated in phosphate-buffered 30% sucrose. Coronal sections at 1.0 to 0.2 mm from bregma were used for immunohistochemical staining and were cut on a freezing microtome (Leica CM3000, Germany) at a thickness of 25 µm.

Immunohistochemistry and immunofluorescence staining

Frozen sections were double-stained for immunofluorescence and cell phenotypes were identified by colocalization of phenotypic markers with BrdU, as previously described, using these primary antibodies (and their concentrations): Goat polyclonal anti-doublecortin (DCX) to label neural progenitor cells (1:100; Santa Cruz); rabbit polyclonal anti-microtubule-associated protein-2 (MAP-2) to label mature neurons (1:50; Cell Signaling); mouse monoclonal anti-βIII tubulin (Tuj-1) to label immature neurons (1:100; Chemicon, Temecula), and rabbit polyclonal anti-caspase-3 to label apoptosis of neurons (1:100; cell signaling). Secondary antibodies: anti-mouse, rabbit, and goat IgG-fluorescein isothiocyanate (FITC) and IgG-Cy3 (1:200; Chemicon). Confocal images were taken with a Zeiss LSM-510 microscope.

Transmission electron microscopy (TEM)

TEM was used to evaluate the ultrastructural changes. Cerebral fragments were fixed with 2.5% glutaraldehyde solution overnight at 4 °C and were washed with PBS after being fixed with 1% osmic acid for 2 h. The samples were embedded in an Epon/Araldite mixture and stained with uranyl acetate and lead citrate, and then were observed under a 1230 type transmission electron microscope (Electron Co, Japan) and photographed.
Western blotting

Rats were sacrificed at 4, 10, and 24 h after reperfusion with an overdose of 3.5% chloral hydrate. Rat cerebral homogenates were collected and centrifuged for 4 min at 3000×g and the resulting supernatants spun at 20000×g for 30 min. Supernatants were diluted into 0.5 mg protein/mL for the measurement of Beclin1 and LC3. Protein content was determined with BSA as a standard according to the Bradford method[25]. Protein samples (20 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA) through electroblotting. Blots were stained with rabbit polyclonal anti-autophagy APG8a (MAP1LC3A) antibody (Abgent, San Diego, CA, USA) and rabbit polyclonal anti-Beclin1 antibody (Cell Signaling). Then the blots were developed by enhanced chemiluminescence using SuperSignal west femto maximum Diego, CA, USA) and rabbit polyclonal anti-Beclin1 antibody.

Measurement of infarct volume

At 14 days after reperfusion, the rats were deeply anesthetized with 3.5% chloral hydrate and then decapitated, after which the brains were rapidly removed. Coronal sections (n=6 for each group) were cut into 2-mm slices and stained with standard 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 10 min at 37 °C followed by overnight immersion in 4% formalin. Infarct volume, expressed as a percentage of whole-brain volume, was measured by an image processing and analysis system (1.25×objective, Q570IW, Leica, Germany) and was calculated by integration of the infarct area in each brain section along the rostral-caudal axis[26].

Neurological deficit scores

Neurological deficit scores were determined at 24 h, 7 days and 14 days after MCAO as previously described[27]: no neurological deficit (value=0); left forelimb flexion when suspended by the tail or failure to extend right forepaw fully (value=1); left shoulder adduction when suspended by the tail (value=2); reduced resistance to lateral push toward the left side (value=3); spontaneous movement in all directions with circling to the left exhibited only if pulled by tail (value=4); circle or walk spontaneously only to the left (value=5); walk only when stimulated (value=6); no response to stimulation (value=7); and stroke-related death (value=8).

Hematoxylin and eosin (H&E) staining

The sections were stained using H&E according to the standard procedures and observed under an Olympus BX51 light microscope. Under a light microscope at 400× magnification, 5 nonoverlapping fields in each of the selected regions of interest (ROIs) were examined to determine the intact neurons and injured neurons.

Data quantification and statistical analysis

Regions of interest (ROIs) were defined as a zone with 250 μm width and 800 μm length in the SVZ, and as a box with 700 μm width and length in the peri-infarct striatum and cortex (Figure 1A). For immunofluorescence counting, four sections were obtained and the results were expressed as the average number per rat. All data were expressed as mean±SD. Statistical analysis was performed using SPSS, version 11.0. ANOVA or a nonparametric test was used for statistical analysis. P<0.05 was considered statistically significant.

Results

Autophagy activation surrounding the ischemic core

Beclin1 has been identified as a mammalian autophagy gene[28]. In the present study, cellular protein extracts from the ipsilateral cortex, striatum and dentate gyrus (DG) surrounding the ischemic core were assessed by Western blot analysis. Here, the expression of Beclin1 was significantly enhanced in all regions as early as 4 h after ischemia (Figure 1B−1D). LC3-II is correlated with the extent of autophagosome formation and LC3-II/LC3-I provides a important molecular marker for detection of autophagic activity[14, 29]. The present study indicated that the LC3-II/LC3-I ratio was significantly increased in the ipsilateral striatum and hippocampus at 4, 10, and 24 h after reperfusion and in the ipsilateral cortex at 24 h after reperfusion, as compared to sham operated animals (Figure 1B−1D). The structures of autophagosomes, bounded by two or more membranes and containing cytoplasmic ground substance or fragments of cellular organelles, were frequently observed in ipsilateral neurons (Figure 2). The results demonstrate that autophagy is activated by transient MCAO.

Beclin1 expression in ipsilateral hemisphere treated with LV-shBeclin1

In order to test whether I/R-induced autophagic injury was dependent on Beclin1 expression, we depleted endogenous Beclin1 levels by stereotaxic injection of LV-shBeclin1 7d before ischemia. Luciferase activity was quantified as the reporter in the present study. At day 3 (preoperative day 4), animals treated with lentiviral vectors containing the luciferase sequence demonstrated positive luciferase expression, which peaked at day 14 (Figure 3C). We showed that LV-shBeclin1 led to a reduction of Beclin1 protein compared with LV-control in targeted regions such as the ipsilateral cortex, striatum and DG in I/R rats (Figure 3A, 3B, 3D). There were no significant differences in the studied parameters between LV-control and LV-shBeclin1 groups (Table 1). In both groups, CBF was reduced to approximately 25% of baseline during MCAO and recovered during the reperfusion phase, reaching 57% and 62% of baseline in the LV-control and LV-shBeclin1 groups, respectively. There were no significant differences in the CBF between the LV-control and LV-shBeclin1 groups (Figure 3E).
Apoptosis of immature neurons surrounding the ischemic hemisphere

Double labeling of active caspase-3 (fragment of p17) and DCX/Tuj-1 (Casp−–DCX+/Casp−–Tuj-1+) was performed in the present study. Activated caspase-3 immunostaining appeared in Tuj-1+ and DCX+ cells in the ipsilateral striatum at 14 days of reperfusion. The Tuj-1+-Casp-3+ and DCX+-Casp-3+ cells were decreased by treatment with LV-shBeclin1 (Figure 4). The results indicated that downregulation of Beclin1 by RNAi reduced the apoptosis of immature neurons in response to reperfusion.

Infarct volume

Transient focal ischemia for 60 min caused infarction in the striatum and frontoparietal cortex on day 14 after reperfusion. Examination of serial brain sections showed that stereotaxic injection of LV-shBeclin1 into the ipsilateral lateral ventricle significantly decreased infarct volume compared with LV-control treatment 14 days after focal ischemia/reperfusion (Figure 5).

Histological outcomes and neurological deficits

In I/R rats treated with LV-control, most neurons were markedly shrunken, and some had pyknotic nuclei and eosinophilic cytoplasm near the boundary of the ischemic core. In the I/R+LV-shBeclin1 group, many neurons were slightly shrunken and surrounded by swollen cellular processes (Figure 6A−6D). Neurological deficit scoring was performed from 24 h to 14 days after reperfusion. Neurological deficit scores
Accumulation, migration and maturity of newborn neurons

We next investigated whether reduction of Beclin1 expression by RNAi participated in neurogenesis in the ischemic brain. Double staining of BrdU and DCX (BrdU⁺-DCX⁺) was used to detect the proliferating neural progenitor cells. Increased number of BrdU⁺-DCX⁺ cells was observed in the subventricular zone (SVZ) 14 days post-ischemia. Beclin1 RNAi increased BrdU⁺-DCX⁺ cells in the ipsilateral SVZ of the MCAO group (Figure 7A−7B). To determine whether Beclin1 RNAi influenced differentiation of neural progenitor cells to neuronal
lineage, we used BrdU- Tuj-1 to detect newborn immature neurons and used BrdU- MAP-2 to detect newborn mature neurons. Consistent with previous reports\cite{30}, we observed that most of these double-staining cells were located at the medial striatum and frontal cortex from the lateral ventricle to the boundary of the ischemic core. Compared with the sham group, BrdU- Tuj-1 cells and BrdU- MAP-2 cells increased

Table 1. Physiological parameters in rats subjected to transient MCAO. n=5. Mean±SD.

| Group                  | MABP (mmHg) | pH     | PCO2 (mmHg) | PO2 (mmHg) |
|------------------------|-------------|--------|-------------|------------|
| Before MCAO            |             |        |             |            |
| LV-control             | 82±6        | 7.35±0.01 | 58±6        | 67±2       |
| LV-shBeclin1           | 89±7        | 7.35±0.01 | 56±3        | 68±5       |
| Ischemia (30 min)      |             |        |             |            |
| LV-control             | 83±3        | 7.35±0.03 | 49±2        | 70±5       |
| LV-shBeclin1           | 85±5        | 7.35±0.02 | 48±5        | 73±7       |
| Reperfusion (30 min)   |             |        |             |            |
| LV-control             | 80±6        | 7.35±0.02 | 45±4        | 79±6       |
| LV-shBeclin1           | 81±4        | 7.35±0.01 | 45±2        | 77±3       |

Figure 4. Apoptosis of new neurons. (A) The merged confocal images confirmed Tuj-1 (red), DCX (red) and caspase-3 (green) expressing cells in the ipsilateral striatum from animals treated with LV-shBeclin1 or LV-control 14 days after MCAO. (B) Quantitative data of each cell. An ANOVA test was used for statistical analysis. *P<0.05, **P<0.01 vs LV-control group. n=6. Data are mean±SD. Scale bar=10 μm.

Figure 5. Infarct volume. (A) Representative TTC staining of the cerebral infarct in comparable sections of rat brain 14 days after ischemia. (B) Quantification of infarct volume. An ANOVA test was used for statistical analysis. *P<0.05 vs LV-control group. n=6. Mean±SD.
Figure 6. Histologic outcomes and neurological deficits. (A) Ipsilateral cortex from I/R rats treated with LV-control: 14 days after reperfusion, most neurons are severely shrunken, and show extensive vacuolation. (B) Ipsilateral cortex from I/R rats treated with LV-shBeclin1: 14 days after reperfusion, some neurons show slightly shrunken perikarya and nuclei. (C) Ipsilateral hippocampus from I/R rats treated with LV-control: 14 days after reperfusion, most neurons are severely shrunken and surrounded by swollen cellular processes and are scalloped in appearance. (D) Ipsilateral hippocampus from I/R rats treated with LV-shBeclin1: many normal neurons and a few dark neurons with perineurial vacuoles are present 14 days after reperfusion. (E) Determination of neurological function from day 1 to day 14 after reperfusion. The scoring scale ranged from 0 to 8. \( n = 10 \). \( ^b P < 0.05, ^c P < 0.01 \) vs I/R+LV-control group. Mean±SD.

Figure 7. Accumulation, migration and maturity of newborn neurons. (A–B) The merged confocal images confirmed DCX expressing cells (green) and BrdU expressing cells (red) in the SVZ of the ipsilateral hemisphere from I/R animals treated with LV-shBeclin1 or LV-control 14 days after MCAO. (C–F) The merged confocal images show BrdU-labeled cells (green) that were co-labeled with Tuj-1 (red; C–D) and MAP-2 (red; E–F) in the ipsilateral cortex of animals treated with LV-shBeclin1 or LV-control 14 days after MCAO. (G–J): Photomicrographs show BrdU-labeled cells (green) that are co-labeled with Tuj-1 (red; G–H) and MAP-2 (red; I–J) in the ipsilateral striatum from animals treated with LV-shBeclin1 or LV-control 14 days after MCAO. (K) Quantitative data of each cell. An ANOVA test was used for statistical analysis. \( ^b P < 0.05, ^c P < 0.01 \) vs I/R+LV-control group. \( n = 6 \). Mean±SD. Scale bar=50 μm in (B) and scale bar=20 μm in (E). SVZ, subventricular zone; Ctx, Cortex; St, striatum; I/R, Ischemia/Reperfusion. LV, lateral ventricle.
in the ipsilateral striatum and frontal cortex 14 days post-reperfusion. Beclin1 RNAi increased the number of BrdU+-Tuj-1+ cells and BrdU+-MAP-2+ cells in the ipsilateral striatum and frontal cortex 14 days after I/R (Figure 7C–J).

Discussion
RNAi-mediated downregulation of Beclin1 decreases apoptosis and promotes neurogenesis in the ischemic hemisphere, and reduces infarct volume and inhibits histological injury in response to transient MCAO. These findings suggest that Beclin1-dependent autophagy may be a target to repair ischemic injury after stroke.

Autophagy is stimulated by nutrient starvation and growth factor deprivation when cells are unable to take up external nutrients. The process involves the rearrangement of subcellular membranes into autophagosomes and autophagic vacuoles[31,33]. Autophagy may be triggered by cerebral ischemia to eliminate damaged mitochondria, thereby aborting apoptosis and delaying necrosis. However, if cerebral reperfusion deficits are not corrected, sustaining a high level of “autophagic stress” will lead to massive lysosomal activation and ultimately cell demise[32]. It was reported that hypoxic/ischemic neuronal death was largely prevented by Atg7 deficiency[39,33]. Induction of autophagy in the ischemic phase was accompanied by activation of AMP-activated protein kinase (AMPK), whereas ischemia/reperfusion stimulates autophagy through a Beclin1-dependent but AMPK-independent mechanism[33]. Here, Beclin1 levels and LC3-II/LC3-I increased together with profuse autophagosomes in the ipsilateral hemisphere in transient MCAO. As previously reported[30], we observed that transient MCAO caused the proliferation of neural progenitor cells and promoted the maturation of newborn neurons in the ipsilateral hemisphere. Interestingly, we found that Beclin1 downregulation by RNAi increased the number of neural progenitor cells (BrdU+-DCX+), newborn immature (BrdU+-Tuj-1+) and mature neurons (BrdU+-MAP-2+) in the surrounding ischemic core of the ipsilateral hemisphere (Figure 7). These findings support the view that dysregulation of Beclin1-dependent autophagy may be linked to cell proliferation[39,40].

Taken together, the data show that inhibition of Beclin1 by RNAi provides neuroprotection, possibly via promotion of neurogenesis and inhibition of apoptosis. Our findings suggest that Beclin1 downregulation could be a therapeutic strategy to improve recovery after stroke.

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Author contribution
Prof Jian-xun LIU designed the research; Dr Yong-qiu ZHENG and Dr Xin-zhi LI performed the research and analyzed the data; Prof Li XU and Yong-gang XU contributed new analytical tools and reagents; and Yong-qiu ZHENG wrote the paper.

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