Original Article

MiR-128-3p activates autophagy in rat brain cells after focal cerebral ischemia reperfusion through targeting Atg1

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Received September 25, 2015; Accepted December 8, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Autophagy is reported to be beneficial for functional recovery in rats after CIR. Increasing evidence suggests that microRNAs are widely involved in rat brains of CIR model. Rat CIR models were established using middle cerebral artery occlusion (MCAO). To evaluate whether the models were successfully established, neurological function score, TTC staining and the water contents were determined. Electron microscopy was applied to explore the presence of autophagosomes. The expression of autophagy-related proteins was determined using Western blot. TargetScan program was used to predict the potential miRs that bind the 3' untranslated region of Atg1. RT-PCR and luciferase assay was applied to explore the level of miRs and the relative luciferase units. The CIR rat models were successfully established through neurological function score, TTC staining and the water contents analysis. Autophagosomes were significantly increased in rat brains of CIR models. Furthermore, the expression of Atg1/pULK1 and LC3II was significantly enhanced in rat brains of CIR models. More importantly, only miR-128-3p was found to be significantly decreased in rat brains of CIR models. Luciferase assay revealed that Atg1 was a target gene of miR-128-3p. To conclude, reduction of miR-128-3p expression contributed to brain cell autophagy mainly by targeting Atg1 after CIR.

Keywords: Cerebral ischemia, reperfusion, autophagy, miR-128-3p, Atg1

Introduction

It is widely reported that stroke significantly leads to death and disability [1]. Thus, early preventions are of particular importance for patients [2]. Obviously, restoration of blood flow contributes to tissue repair and functional recovery. However, it may leads to cerebral ischemia reperfusion (CIR), such as oxidative stress, inflammation and apoptosis [3, 4].

After ischemic stroke, the balance between cell survival and death is disturbed [5]. Autophagy is reported to prompt cell survival and can be activated by various factors, including oxidative stress and endoplasmic reticulum stress [6, 7]. Researchers have found that autophagy is significantly enhanced after cerebral ischemia [5]. At present, the expression of LC3II and Beclin 1 was found to be significantly increased in the rat brains after cerebral ischemia [8]. Thus, it is important to explore the specific mechanism through which autophagy is activated.

MicroRNAs (miRs) are small non-coding RNAs with about 22 nucleotides [9, 10]. Through incomplete pairing mechanism, they can repress target gene expression [11]. After cerebral ischemia, abnormal miRNA expression has been widely identified. For instance, it was reported that inhibition of miR-155 enhanced functional recovery after stroke in mice [12]. In addition, miR-107 was found to be involved in post-stroke angiogenesis mainly by targeting Dicer-1 [13]. In this study, we explored the miRNAs that regulated the expression of Atg1, a key protein involved in autophagy. And found only miR-128-3p was significantly decreased. Further study revealed that decreased miR-128-3p expression in brain cells contributes to autophagy activation after cerebral ischemia in rats.
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Materials and results

Animals

Adult male Sprague-Dawley rats (220-230 g) was purchased from the Health Science Center of Peking University (Beijing, China) and housed in the laboratory animal room. The rats were maintained at 25°C ± 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 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 XXXX.

Transient MCAO

Rats were subjected to transient focal cerebral ischemia induced by right MCAO as previously described [14].

Assessment of neurological deficit score and analysis of survival rates

The neurological deficit score was evaluated before rats were sacrificed 24 h after reperfusion as described previously [15]. Each rat was evaluated by 2 examiners. The following neurological deficit scoring (NDS) system was used: 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); and 4, no spontaneous locomotor activity or barrel rolling (critical). If no deficit was observed 2 h recovering from anesthesia, the animal was removed from further study.

Edema measurement

Rats were decapitated under deep anesthesia with 10% chloral hydrate at 6 h, 12 h, 24 h, and 72 h of reperfusion. The ipsilateral and contralateral hemispheres were dissected and the wet weight of the tissue was determined. The tissues were dried at 120°C for 24 hours. The percent cerebral water was determined as (wet weights - dry weights)/dry weights × 100.

Measurement of infarct volume

After reperfusion, the rats were deeply anesthetized with 3.5% chloral hydrate and then decapitated, after which the whole brains were rapidly removed. Coronal sections (n = 10 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, Wetzlar, Germany) and was calculated by integration of the infarct area on each brain section along the rostral caudal axis (30).

Transmission electron microscopy (TEM)

TEM was used to evaluate the ultrastructural change of brain sections. Cerebral fragments were fixed with 2.5% glutaraldehyde solution overnight at 4°C; then they were washed with PBS and fixed with 1% osmic acid for 2 h. Tissues were embedded in an Epon/Araldite mixture. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. The samples were observed under a 1230 type transmission electron microscope (Electron Co., Tokyo) and photographed.

Protein extraction, western blotting and antibodies

Tissue or cell protein was extracted using RIPA buffer (SolarBio, Beijing, China). The proteins were resolved by 10% SDS-PAGE gel and transferred onto a PVDF membrane. The protein was detected with primary antibodies (Atg1, pULK, LC3) overnight at 4°C. GAPDH was used as the internal control. Signals were explored with enhanced chemiluminescence according to the manufacturer’s instructions (Millipore, Billerica, MA, USA).

RNA extraction

Total RNA were extracted from gastric tissues or cells using TriZol reagent according to the manufacturers’ instructions (Life Technologies, Carlsbad, CA, USA).

Reverse transcription quantitative polymerase chain

To quantify the amount of miRNA, total RNA were reverse transcribed using Takara MicroRNA Reverse Transcription Kit (Takara) with specific primers for miR-132-3p and U6.
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Subsequently, the PCR amplification was performed. 1 ml of cDNA was used for qPCR using SYBR green Master mix (Roche, Basel, Switzerland) on a Roche light cycler 480 at: 95°C, 10 min; 50 cycles of 95°C for 10 sec, specific annealing temperature for 10 sec, 72°C for 5 sec; 99°C for 1 sec; 59°C for 15 sec; 95°C for 1 sec; cooling to 40°C. Relative miRNA expression of miR-132-3p was normalized against the endogenous control, U6, using the delta-delta CT method.

Bioinformatics predictions

To determine the potential miRNAs that target MUC13, bioinformatics prediction was performed using TargetScan (http://www.targetscan.org).

Luciferase reporter assay

The 3’untranslate region of Atg1 was cloned into the pmirGLO plasmids. Dual luciferase reporter assay was conducted according to the manufacturer’s instructions (Dual-Luciferase® Reporter Assay System/Dual-Luciferase®, Promega).

Statistical analysis

The data were expressed as the mean ± SEM. The number of independent experiments was represented by “n”. Multiple comparisons were performed using one-way ANOVA followed by Tukey’s multiple-comparison test, where P<0.05 was considered significant.

Results

Establishment of cerebral ischemia rat model

As shown in Figure 1A, MCAO significantly enhanced infarction rat brain cortex. Meanwhile, the neurological deficits were obviously increased in the cerebral ischemia model compared with sham control (Figure 1B). And brain edema was also aggregated after cerebral ischemia (Figure 1C), suggesting the successful
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Autophagosomes (APs) and autolysosomes (ALs) were increased after CIR.

Enhanced Atg1 expression in CIR rat brains

Atg1/pULK1 complex was reported to be involved at the initial stage of autophagy [16]. Thus in this study, we explored their expression after CIR. As shown in Figure 3, the expression of Atg1/pULK1 was significantly increased after CIR in a time dependent manner. Furthermore, we explored the expression of LC3II, a key indicator of autophagy. Obviously, LC3II was increased in CIR model. The data confirmed that autophagy was enhanced after CIR.

Decreased miR-128-3p expression enhanced autophagy by targeting Atg1

To explore the specific miRNAs that regulate the expression of Atg1, the potential miRs possibly binding the 3’untranslated region (UTR) of Atg1 were analyzed by TargetScan. As shown in Figure 4A, eight miRNAs were found to bind the 3’UTR of Atg1, including miR-17-5p, miR-20-5p, miR-93-5p, miR-106b-5p, miR-105, miR-291-3p, miR-142-5p and miR-128-3p. RT-PCR revealed that only miR-128-3p was found to be significantly decreased in the rat brains after CIR (Figure 4B). The 3’UTR of Atg1 was cloned into the pmirGLO vector. Dual luciferase assay revealed that miR-128-3p could significantly decreased the relative luciferase activity in HEK 293 T cells transfected with pmirGLO-Atg1-3’UTR compared with blank vector (Figure 4C). These date indicated that the reduced miR-128-3p expression contributed to autophagy.
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Discussion

In the present study, we demonstrate that autophagy was significantly enhanced after CIR in rat brains. We demonstrated the enhanced expression of autophagy-related proteins, including Atg1/pULK1 and LC3II. More importantly, we explored the miRNAs that potentially regulate the expression of Atg1 and found only miR-128-3p was decreased in rat brains of CIR model. Dual luciferase assay revealed that miR-128-3p could significantly decreased the relative luciferase activity in HEK 293 T cells transfected with pmirGLO-Atg1-3'UTR compared with blank vector. n = 6 animals per group. *P<0.05 vs. sham group S; ***P<0.001 vs. blank vector pmirGLO.

Ischemic cerebrovascular disorder is increasingly become a severe public health problem, which cause great inconvenience for patients and bring heavy economic burdens for their families [17]. From this perspective, it is important to prevent and treat ischemic cerebral disease [18, 19]. At present, restoration of blood circulation and removing blood stasis are the two most common treatment methods. However, the symptoms of some patients may be even aggravated after the restoration of blood, which is referred as CIR. How to prevent CIR-related injury has become a hot research topic for clinicians.

Recent studies have found that autophagy contributes to the functional recovery of brains after CIR [20, 21]. As an intracellular degradation system, autophagy mainly functions to maintain cell homeostasis by recycling intracellular materials [22]. In the process of autophagy, cytoplasmic components are incorporated into autophagosomes and can be further hydrolyzed by lysosomal hydrolases. There are four stages that are involved in autophagy, including induction, initiation, extension and maturity [23]. For autophagosome formation, 6 functional groups are identified, including Atg8 and Atg12 complex, the autophagy specific PI3K complex, the Atg2-Atg18 complex and Atg9-Atg1 complex [22]. Among these complex, Atg1 is the most upstream factor and it is strongly initiates the formation of autophagosome [24, 25]. Thus, understanding the molecular mechanism in which Atg1 is regulated is of great importance.

MiRs are widely reported to be aberrantly expressed in CIR animal modes. For instance,
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MicroRNA-9 was found to induce the defective trafficking of Nav1.1 and Nav1.2 through targeting Navβ2 in rat with chronic brain hypoperfusion [26]. Furthermore, miR-29b is suggested to be a potential therapeutic target in cerebral ischemia associated with aquaporin 4 [27]. In this study, we made a systematic analysis of miRs that potentially regulate the expression of Atg1. And we found miR-128-3p was significantly decreased in rat brains of CIR model. Further study revealed that Atg1 was a target gene of miR-128-3p. Our study suggested that miR-128-3p participated in the initiation of autophagy mainly by regulating the Atg1 kinase.

In summary, we show novel evidence that autophagy was significantly enhanced in rat brains after CIR. Furthermore, reduction of miR-128-3p expression contributed to brain cell autophagy mainly by targeting Atg1 after CIR.

Disclosure of conflict of interest

None.

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