Original Paper

Mitofusin 2 Exerts a Protective Role in Ischemia Reperfusion Injury Through Increasing Autophagy

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Key Words

Autophagy • Cerebral ischemia reperfusion injury • Mitofusin 2 • Autophagic flux • Chloroquine

Abstract

Background/Aims: Autophagy is essential for maintaining cellular homeostasis and the survival of terminally differentiated cells as neurons. In this study, we aim to investigate whether mitofusin 2, a mitochondrial fusion protein, mediates autophagy in cerebral ischemia/reperfusion (I/R) injury. **Methods:** Primary cultured neurons were treated with oxygen-glucose deprivation/reperfusion to mimic cerebral I/R injury *in vitro*. Autophagosomes were visualized upon TEM. Autophagy-markers were then detected to monitor autophagy by western-blot and real-time PCR, and the autophagic flux was tracked with a mRFP-GFP-LC3 construct by fluorescence as well as autophagy inhibitors and agonists. The up- and downregulation of Mfn2 were through transfecting a lentivirusexpression vector respectively. And neuronal injury was detected by cell counting kit and TUNEL assay. **Results:** Results showed I/R increased autophagosome formation and inhibited autolysosome degradation. Furthermore, use of autophagy related agents demonstrated that I/R injury was caused by insufficient autophagy and aggravated by impaired autophagic degradation. The results also indicated that mitofusin 2 could ameliorate I/R injury through increasing autophagosome formation and promoting the fusion of autophagosomes and lysosomes. In contrast, downregulation of mitofusin 2 aggravated the I/R injury by inhibiting autophagosome formation and the fusion of autophagosomes and lysosomes. Additionally, mitofusin 2 overexpression did not lead to autolysosome accumulation induced by I/R. **Conclusions:** In summary, this study explicitly demonstrated that mitofusin 2 could ameliorate I/R injury mainly through promoting autophagy, which represented a potential novel strategy for neuroprotection against cerebral I/R damage.

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Introduction

Cerebral ischemia reperfusion (I/R) injury is the third leading cause of death in industrialized countries. It often leads to permanent disability with extensive damage to the cerebral tissue [1]. Blood reperfusion, which follows cerebral ischemia, often results in inflammation, oxidative stress and endoplasmic reticulum stress, which can induce autophagy [2]. Autophagy is an intracellular degradation system that transports cytoplasmic constituents to lysosomes for degradation. The exact role of autophagy in I/R injury is still controversial [3, 4]. Recent studies demonstrated that autophagy played a critical role in neuronal survival and death. Furthermore, studies suggested that autophagy could be induced by I/R injury in the liver, kidney, heart and brain [5-7]. Even though the mechanisms that underpin the role of autophagy in cerebral I/R injury are not completely understood, it seems that reperfusion may be the critical point for I/R related autophagy.

There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy (hereafter referred to as autophagy) is the most common type of autophagy. In autophagy, autophagosomes (double-membrane vacuoles) transport cytoplasmic content into lysosomes (autolysosomes) for degradation of its cargo [8, 9]. This catabolic process is mediated by a number of autophagy and autophagy-related proteins, including microtubule-associated protein light chain 3 (LC3), Beclin 1, and Nucleoporin p62 (p62). LC3, exists in two forms LC3 I and LC3 II, is a widely used marker for autophagy and is necessary for the formation of autophagosomes and the autophagosomal membrane [10]. And the transformation of LC3 I into LC3 II is associated with the formation of autophagosomes. Therefore, the accumulation of LC3 II is indicative of increased autophagosome numbers or an autolysosome influx. Beclin 1, a component of the phosphatidylinositol 3-kinase (PI3K) complex, plays a key role in the early stages of autophagosomal membrane formation [11]. The adaptor protein p62 is required for the formation of ubiquitinated protein aggregates and is degraded through the autophagy-lysosomal pathway. This degradation is mediated by the interaction of p62 with both ubiquitinated proteins and LC3, which facilitates the delivery of these protein which aggregated to the autophagy system [12]. In summary, Beclin-1 and LC3 II are involved in the formation of autophagosomes and their accumulation is indicative of increased autophagy, while p62 is degraded constantly by autophagy and therefore its accumulation indicates a decrease in autophagic flux [8]. Although the expression level of LC3 II is low in the brain, the existence of basal autophagy in neurons and its significance have been previously demonstrated [13].

Mitofusins 1/2 (Mfn1, 2) are mitochondrial membrane proteins that are essential for mitochondrial fusion [14, 15]. Mfn2 played a role in maintaining mitochondrial function under stress [16]. Furthermore, it also modulated cellular processes through several molecular pathways, like PI3K/Akt, Bcl-2/Bax, and antiviral signaling [17-19]. Recently, it was reported that mitochondria supply membranes for autophagosome biogenesis during starvation and that Mfn2 was crucial for this process [20]. Another recent study reported that Mfn2 participated in parkin-dependent autophagy, which was thought to mediate the removal of damaged mitochondria [21]. Our previous study demonstrated that Mfn2 played a protective role in hypoxia via the restoration of mitochondrial function and the inhibition of apoptosis [22]. In this study we aimed to demonstrate the role of Mfn2 in cerebral I/R injury in primary cortical neurons by investigating the relationship between Mfn2 and autophagy in mechanisms of I/R injury. The results indicated that Mfn2 could ameliorate the effects of cerebral I/R injury by modulating autophagic flux.

Materials and Methods

Cell cultures and I/R procedures

Primary cultured cortical neurons were prepared from embryonic day (E) 14-15 C57BL/6 mouse embryos as previously described [23]. Briefly, embryos were extracted from pregnant females, and their

cortices were dissected and minced. Thereafter, they were incubated for 30 min at 37 °C in Ca²⁺/Mg²⁺-free HBSS containing 15 U/mL papain solution (Worthington, Lakewood, NJ, USA) and 0.2 mg/mL DNase I (Beyotime, Haimen, China). Then, the cells were re-suspended in a neurobasal medium supplemented with 2% B27 supplement and 0.5 mM L-GlutaMax™-I (Gibco, NY, MD, USA) and plated onto poly-L-lysine (Sigma-Aldrich, MO, USA) coated dishes at a density of 2.5×10^5 cell/cm². After 7-10 days of culture, cells were used for experiments. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional of Animal Care and Use Committee of the Fourth Military Medical University.

For I/R treatment, cells were rinsed once with warm glucose-free Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, MD, USA) and then refreshed with warm glucose-free DMEM. Cells were then immediately placed into a hypoxia chamber loaded with mixed gas containing 5% CO₂ and 95% N₂. Neurons were incubated at 37 °C in the hypoxia chamber for 3 h before reperfusion [24]. Neurons in the control group were always maintained in normoxic incubation conditions. For the reperfusion phase, neurons were refreshed with normal culture medium for pre-defined time points. Then, 3-methyl adenine (3-MA, 5 mM), an E64d and pepstatin A mixture (E64d, 25 μM; Pep A, 25 μM), and rapamycin (100 nM) or chloroquine (CQ, 10 mM) (Sigma-Aldrich, MO, USA) were dissolved in normal neurobasal medium and added to the cells at the onset of reperfusion.

Lentivirus infection

For the down-regulation of Mfn2, short hairpin RNA (sh-Mfn2, NM_001285920) was subcloned into a lentivirus (LV) expression vector (GeneChem Company, Shanghai, China). For Mfn2 overexpression, a LV-Mfn2 overexpression system (NM_001285920) was developed in an Ubi-3FLAG-MCS-IRES-puromycin construct (GeneChem Company, Shanghai, China). Neurons were transfected with the virus vectors (multiplicity of infection; MOI = 20) for 48-72 h. The neurons were then used for different experimental studies. LV-Scr and sh-Scr were used as negative controls.

Transmission electron microscopy

To visualize autophagosomes and autolysosomes, neurons were analyzed under transmission electron microscopy (TEM). Samples were concentrated at 1000 r/min, and fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) for 3 h at 4 °C. They were washed five times with 0.1 M cacodylate buffer containing 0.1% CaCl₂. Samples were then postfixed with 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% CaCl₂ for 1 h. After rinsing with cold PBS, cells were dehydrated slowly in an ethanol series and propylene oxide at 4 °C. After resin polymerization at 55 °C for 24-36 h, serial sections were cut with a diamond knife and mounted on formvar-coated slot grids. Finally, high resolution pictures were captured.

Fluorescence of mRFP-LC3 and mRFP-GFP-LC3

In order to detect and quantify autophagosome/early autophagic vacuoles, late/degradative autophagic vacuoles, and autolysosomes, fluorescent mRFP-LC3 and mRFP-GFP-LC3 virus constructs (Invitrogen, Carlsbad, CA, USA) were used. Neurons were transfected with the different virus vectors for 24-48 h followed by additional experimental studies. The samples were then examined under a confocal microscope (FV10i, Olympus, Tokyo, Japan) to detect the LC3 fluorescence puncta.

Cell viability

Neurons were cultured in 96-well plates for I/R exposure. The cells were analyzed with a cell counting kit-8 (CCK-8) (Dojindo, Japan) following the manufacturer's protocol. After 2 h of incubation, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability in different groups was expressed as a percentage of the control group.

TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining method was employed to detect cellular apoptosis according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). Briefly, neurons were cultured on plates and subjected to I/R. Neurons were subsequently washed with PBS and fixed with 4% paraformaldehyde for 30 min. Neurons were then incubated with a prepared mixture of working liquid at 37 °C for 1 h in the dark. Prior to detection, nuclei were stained with Hoechst

33342 (Sigma Aldrich, St. Louis, MO, USA) for 10 min and then repeatedly washed with PBS. The positive control groups were treated according to the manufacturer's instructions. After staining, neurons were examined under a confocal microscope (FV10i, Olympus, Tokyo, Japan) to observe the green fluorescence levels. Experiments were performed in triplicate, and the percentage of positive cells was determined (TUNEL positive cells/Total cells).

Western blot

An equal amount of protein (20 µg) per sample was separated by SDS-PAGE and transferred to nitrocellulose membranes. The nonspecific binding of antibodies was blocked with 5% non-fat dried skim milk in PBS with 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with the appropriate primary antibodies (Mfn2 1:1000, Caspase 3 1:1000, Cleaved-caspase 3 1:1000, β-actin 1:5000, LC3- II 1:1000, Beclin-1 1:2000, p62 1:2500, Cell Signaling Technology, Danvers, MA, USA), as previously described [25].

RNA extraction and real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. After extraction, 500 ng of the total RNA was used as a template to synthesize cDNA using a first strand synthesis kit (Takara, Dalian, China). Then, cDNA was used in quantitative real-time PCR analysis. The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: forward, 5'-GGG TCA GAA GGA TTC CTA TG-3'; reverse: 5'-GGT CTC AAA CAT GAT CTG GG-3'. Primers used for Mfn2 were as follows: forward 5'-TGA AGG CCT GAG TTA ATA CCT AGC-3'; reverse: 5'-TCA TCA ACT AAG TGA CAA GGA AGG-3'. Beclin-1 primers were: 5'-GGG TCA CCA TCC AGG AAC TCA-3'; reverse: 5'-CAC CAT CCT GGC GAG TTT CA-3'. Data were analyzed using a comparative critical threshold (Ct) method in which the amount of the target gene was normalized to the amount of endogenous control and relative to the control samples.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA). Data were presented as the mean ± standard error of the mean (SEM), and significant differences between groups were assessed using one-way or two-way analysis of variance (ANOVA) followed by a serious comparison or different multiple comparison. All experiments were performed at least three times.

Results

Autophagy was activated by I/R injury in cultured cortical neurons

To study I/R-induced autophagy, cultured cortical neurons were exposed to oxygen-glucose deprivation reperfusion in an *in vitro* model of cerebral I/R. The protein expression of the autophagic markers LC3 II and Beclin 1 were detected by western blotting 6, 12, 24, 36 and 48 h after reperfusion. The results showed that LC3 II and Beclin 1 levels both increased at different reperfusion time points over a 48 h period (Fig. 1a, b, c). The expression of LC3 II was more significantly elevated 36 h post-reperfusion ($P < 0.001$). Real-time PCR results also showed similar increased levels of Beclin 1 mRNA (Fig. 1d). The results of TEM analysis, a standard technique used for autophagy detection, indicated an increase in autophagosomes and autolysosomes (red arrows) filled with cargo in I/R-treated neurons 36 h post-reperfusion (Fig. 1e). LC3 puncta decorating autophagic vesicles were commonly used to monitor the occurrence of autophagy. In this model, the increased number of mRFP-LC3 puncta confirmed activated autophagy after I/R (Fig. 1f). These results indicated that I/R treatment activated the initiation of autophagy, which came in agreement with previous reports [26].

Increased autophagosomes formation and inhibited autophagic degradation were induced by I/R

The detection of autophagic flux, a dynamic process (autophagosomes formation and autophagic degradation), would be important for monitoring the autophagy process.

Autophagic flux could be defined as the difference between LC3 II protein levels in the absence and presence of lysosomal inhibitors. Chloroquine (CQ) could increase lysosomal pH which lead to the inhibition of lysosomal hydrolase activity and hinders the fusion of lysosomes with autophagosomes. Therefore, application of CQ was used to track the autophagic flux after I/R injury. Starting from 12 h post-I/R injury, the levels of LC3 II were significantly higher than that in the control groups in the absence or presence of CQ (Fig. 2a, b). Collectively, these results as well as the increased expression of Beclin 1 suggested that the formation of autophagosomes were increased with I/R injury.

However, based on the aforementioned results, there was not enough evidence to confirm the inhibition of autophagic flux in I/R. Stimulation of autophagic flux could cause the depletion of SQSTM1/p62, along with other autophagic substrates. Therefore, upon the impairment of autophagic clearance, p62 would be accumulated within cells [27]. Our results showed that the protein levels of p62 decreased in the early phase before increasing starting from 24 h to 48 h post-reperfusion (Fig. 2c, d). This indicated that I/R injury could inhibit autophagic degradation. To confirm this theory, direct image analysis of LC3 II fluorescence was performed. The tandem-tagged LC3 construct, mRFP-GFP-LC3, was applied to distinguish early phase and late phase autophagosomes (autophagosomes and autolysosomes). The GFP-tag was quenched quickly in the acidic environment of the lysosomes, leaving only the mRFP-tag detectable. Therefore, the red puncta overlaying the green ones, which appeared

Fig. 1. Autophagy was activated by I/R injury in cultured cortical neurons. Primary cultured cortical neurons were incubated in a hypoxia chamber for 3 h, followed by a 48 h reperfusion period. (a, b) Western blot and quantification analysis of LC3 II detected at indicated time points during the reperfusion period. (a, c) Western blot and quantification analysis of Beclin 1 detected at indicated time points during the reperfusion period. (d) mRNA of Beclin 1 was quantified by real-time PCR analysis. (e) Autophagosomes and autolysosomes were viewed under TEM. (f) mRFP-LC3 puncta were acquired by fluorescence. Results from three independent experiments are shown. The data represent the mean $\pm$ SEM (one-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control groups.

Fig. 2. Increased autophagy activity and inhibited autophagic degradation induced by I/R. (a, c) Western blot analysis of LC3 II and p62 were detected at the indicated time points during the reperfusion period. (b, d) Quantitative analysis of LC3 II and p62 (n=4). The data represent the mean $\pm$ SEM (one-way ANOVA and two-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with Con-CQ(-) or Con. ## $P < 0.01$; ### $P < 0.001$ compared with Control-CQ(+).

yellow in merged images, were indicators of early autophagosomes; while the free red puncta that did not overlay the green ones, and appeared red in merged images, were indicators of autolysosomes. Following I/R injury, results clearly showed that the numbers of autolysosomes (free red puncta) were significantly higher in the I/R groups than the control group (1.0 ± 0.5774 vs 17.33 ± 1.856 , $P < 0.001$) (Fig. 3a, b). And we examined the influence of rapamycin (a well-documented autophagy inducer) on the number of autophagosomes and autolysosomes [28]. Our results demonstrated that the numbers of yellow puncta (early autophagosomes) were higher in the rapamycin treated group than the control group (2.0 ± 0.5774 vs 6.667 ± 0.8819 , $P < 0.05$) (Fig. 3a, b). Furthermore, the results indicated that the accumulation of mRFP-LC3 II puncta coalesced to the lysosomes increased after I/R, however, there was no significant difference in the numbers of early autophagosomes (yellow puncta) among the groups examined (Fig. 3a, b). Additionally, the numbers of autolysosomes (free red puncta) were higher in the I/R groups compared to the rapamycin group (4.667 ± 1.202 vs 17.33 ± 1.856 , $P < 0.01$). In contrast, the number of early autophagosomes (yellow puncta) were reduced in the I/R group compared to the rapamycin group (6.667 ± 0.8819

Fig. 3. Primary cultured cortical neurons were incubated in a hypoxia chamber for 3 h, followed by a 36 h reperfusion period. CQ or a mixture of E64d/Pep A were added to the neurons at the onset of reperfusion. (a) Fluorescence of mRFP-GFP-LC3 was detected by confocal microscopy in different conditions. (b) The number of autophagosomes or autolysosomes was counted per neuron, and quantification analysis is presented (over 200 cells were counted for each individual group; $n = 3$). The data represent the mean $\pm$ SEM (two-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The red line: significant difference of autophagosomes numbers in two groups. The yellow line: significant difference of autolysosomes numbers in two groups.

Fig. 4. Inhibited autophagic flux aggravated neuronal cell death induced by I/R. Primary cultured cortical neurons were incubated in a hypoxia chamber for 3 h, followed by a 36 h reperfusion period. 3-MA, CQ or a mixture of E64d/Pep A or Rapa were added to the neurons at the onset of reperfusion. (a) TUNEL was used to detect neuronal apoptosis after I/R in the different groups. (b) Quantification analysis of TUNEL results (over 1000 cells were counted for each individual group) ($n = 4$). (c) Analysis of cell viability was detected by CCK-8. Results from four independent experiments are shown ($n = 4$). The data represent the mean $\pm$ SEM (one-way ANOVA). ** $P < 0.01$ compared with the untreated neurons. # $P < 0.05$; ## $P < 0.01$ compared with I/R groups.

vs 3.0 ± 0.5774 , $P < 0.05$) (Fig. 3a, b). This indicated that other mechanisms might also contribute to the accumulation of free red puncta (autolysosomes) after I/R. In addition, autophagy inhibitors CQ and E64/Pep A were used to investigate the cause of autolysosome accumulation in I/R. CQ and E64/Pep A were known to inhibit autolysosome degradation. Treatment of the I/R group with CQ (I/R+CQ group) resulted in increased accumulation of early autophagosomes (yellow puncta), while increased accumulation of autolysosomes (free red puncta) were observed in the I/R+E64/Pep A group. Combining these results, the increased p62 expression and autolysosome accumulation indicated that I/R injury could lead to impaired autophagic degradation.

Impaired autophagic flux aggravated neuronal injury induced by I/R

To study the role of autophagy in I/R, neuronal injury was examined with the addition of several autophagy inhibitors and agonists. In agreement with previous studies, incubation with the autophagy inhibitor 3-MA in the reperfusion phase triggered enhanced neuronal injury after I/R, as demonstrated by the CCK-8 ($P < 0.01$) and TUNEL ($P < 0.01$) assay results (Fig. 4a, b, c) [26]. Additionally, CQ and E64/Pep A were applied to investigate the role of inhibited autophagic degradation in neuronal injury under I/R conditions. Results demonstrated that both CQ and E64/Pep A aggravated the neuronal injury after I/R, as indicated by the increased apoptosis rate (TUNEL assay) ($P < 0.01$; $P < 0.01$) (Fig. 4a, b) and decreased cell viability (CCK-8 assay) ($P < 0.05$; $P < 0.01$) (Fig. 4c). These results indicated that the inhibited autophagosome formation and the impaired autophagic degradation could aggravate the neuronal injury induced by I/R. On the other hand, addition of rapamycin, resulted in decreased apoptosis ($P < 0.05$) and enhanced the cell viability ($P < 0.05$), thus indicating its protective role in I/R injury (Fig. 4a, b, c). These results indicated that autophagy might play a protective role in I/R, and its inhibition in the initiation or the degradation phases could aggravate neuronal injury induced by I/R.

Protective role of Mfn2 in I/R injury

In the reperfusion phase, the expression of Mfn2 mRNA and protein were mildly elevated in the early phase (up to 24 h), and then significantly decreased after 36 h and 48 h compared to the control group (Fig. 5a, b), which indicated the changes in transcription of Mfn2. Further to investigate the role of Mfn2 in I/R injury, lentivirus vectors for Mfn2 overexpression (LV-Mfn2) and short hairpin RNA for Mfn2 knock-down (sh-Mfn2) were used (Fig. 5c). Whether beneficial or detrimental role of Mfn2 was determined by neuronal injury assays. Results indicated that overexpression of Mfn2 significantly increased cell viability ($P < 0.05$) (Fig. 5e) and decreased TUNEL positive staining ($P < 0.05$) (Fig. 5f) and cleaved caspase 3 levels ($P < 0.05$) (Fig. 5d), after I/R injury (36 h post-reperfusion). In contrast, silencing of Mfn2 aggravated the I/R injury, as evidenced by the decreased cell viability ($P < 0.05$) (Fig. 5e)

and the increased apoptosis rate, verified by TUNEL staining and increased cleaved caspase 3 ($P < 0.05$, $P < 0.01$, respectively) (Fig. 5f, 5d). These results demonstrated that Mfn2 played a protective role in I/R injury, and deletion of Mfn2 would aggravate I/R injury.

The protective role of Mfn2 in I/R might through promoting autophagy

In present study, we observed the protective roles of both autophagy and Mfn2 in I/R injury. Therefore, we investigated the possible relationship between autophagy and Mfn2. Western blot analysis showed that Mfn2 overexpression triggered a significant increase of LC3 II in both the control and I/R groups in the presence of CQ (6.706 ± 0.5586 vs 8.20 ± 0.7567 , $P < 0.05$, con) (8.072 ± 0.7448 vs 10.52 ± 0.6994 , $P < 0.05$, I/R) (Fig. 6a). This indicated that Mfn2 played a role in promoting autophagosome formation under normal or I/R injury conditions. Additionally, the silencing of Mfn2 by sh-Mfn2 was applied to investigate the role of endogenous Mfn2 in I/R-induced autophagy. The expression of LC3 II in the sh-Mfn2 transfected neurons was not significantly different from its expression in the control group under normal and I/R conditions. However, by tracking the autophagic flux with CQ, it was observed that the LC3 II level significantly decreased in the sh-Mfn2 transfected neurons upon CQ treatment in the control and I/R groups (6.706 ± 0.5586 vs 3.742 ± 0.3555 , $P < 0.01$, con) (8.072 ± 0.7448 vs 4.687 ± 0.4297 , $P < 0.01$, I/R) (Fig. 6a). This result suggested that Mfn2 might be an indispensable factor in autophagosome formation and implied that the protective role of Mfn2 in I/R injury could be mediated through promoting autophagy.

An increase in autolysosome accumulation could aggravate the neuronal injury induced by I/R. Present results showed that Mfn2 could increase autophagy, therefore, it was critical to investigate whether the overexpression of Mfn2 increased the accumulation of autolysosomes in I/R or not. Followed by, we explored if overexpression of Mfn2 increased the accumulation of autolysosomes in I/R by detecting p62. The results showed that the overexpression of Mfn2 had no apparent effect on the regulation of p62 under control or I/R conditions (Fig. 6b). In contrast, silencing Mfn2 significantly increased the accumulation of p62 in the control and I/R groups (1.0 ± 0.1007 vs 1.574 ± 0.0899 , $P < 0.01$, con) (1.455 ± 0.0712 vs 2.156 ± 0.0753 , $P < 0.01$, I/R) (Fig. 6b), which indicated that Mfn2 could contribute to autophagic flux.

In order to further explore the role of Mfn2 in autophagic flux induced by I/R injury, cultured neurons were examined under a confocal microscope following mRFP-GFP-LC3 infection. Results confirmed that the deficiency of Mfn2 significantly decreased the total number of puncta (2.0 ± 0.5774 vs 8.667 ± 1.453 , $P < 0.05$) compared to the control groups

Fig. 5. Protective role of Mfn2 in I/R injury. Neurons were infected with the specific virus vector for 48–72 h followed by induction of the I/R model. (a) Western blot and quantification analysis of Mfn2 at indicated time points during the reperfusion period ($n = 4$). (b) Mfn2 mRNA was detected by real-time PCR ($n = 4$). (c) The efficiencies of LV-Mfn2 and shMfn2 were identified by western blotting ($n = 4$). (d) The total caspase 3 and cleaved caspase 3 were detected by western blotting after reperfusion in the different groups ($n = 4$). (e) Cell viability was detected by CCK-8 ($n = 4$). (f) TUNEL analysis was used to assess the neuronal injury ($n = 4$). The data represent the mean $\pm$ SEM (one-way ANOVA; two-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the untreated neurons. # $P < 0.05$; ## $P < 0.01$ compared with I/R groups.

(Fig. 6c, d). Following I/R injury, the knock-down of Mfn2 with sh-Mfn2 resulted in a significant decrease in the total number of fluorescent puncta (27.67 ± 2.603 vs 15.67 ± 0.333 , $P < 0.01$) compared with the I/R control group, however, almost all of the fluorescent puncta were yellow puncta in the I/R+sh-Mfn2 group (14.0 ± 1.155 vs 2.333 ± 0.333 , $P < 0.01$) (Fig. 6c, d). This indicated that the deficiency of Mfn2 could decrease autophagosome formation and inhibit the fusion of autophagosomes and lysosomes. Interestingly, under I/R conditions, Mfn2 overexpression significantly increased the total number of fluorescent puncta (27.67 ± 2.603 vs 44.0 ± 3.512 , $P < 0.05$) and yellow puncta (2.333 ± 0.333 vs 9.667 ± 2.028 , $P < 0.05$) compared with the I/R group (Fig. 6c, d). However, there was no significant difference in the number of free red puncta between the two groups. These results indicated that Mfn2 could increase autophagosome formation and promote the fusion of autophagosomes and lysosomes, without aggravating the I/R induced accumulation of autolysosomes.

Discussion

Autophagy is an energy-dependent process that degrades and recycles subcellular organelles and metabolites [29]. It plays a pivotal role in maintaining cellular homeostasis which is essential for normal cellular function and survival of terminally differentiated cells such as neurons. Over the last decade, autophagy had been implicated in several pathological conditions, including cancer, autoimmune diseases, neurodegeneration and aging [30-32]. In the present study we aimed to identify the exact role of autophagy in I/R injury and further to investigate the role of Mfn2 and autophagy in I/R *in vitro*. Firstly, we comprehensively demonstrated changes in autophagic flux after I/R injury. Then, the exact roles of autophagy and Mfn2 in I/R were explored through assessing neuronal injury. Finally, with the aid of LV-Mfn2 and sh-Mfn2, we demonstrated that the protective role of Mfn2 in I/R was achieved through increasing autophagosome formation without increasing autolysosome accumulation induced by I/R injury.

In agreement with previous investigations, the increased LC3 II levels and mRFP-LC3 puncta numbers, as well as the appearance of autophagosomes, confirmed that autophagy was activated in the reperfusion phase in the primary cultured cortical neurons [33]. Autophagy is a highly dynamic, multi-step process that involves two processes both could promote autophagosome accumulation (increased expression of LC3 II), namely an increase in the rate of autophagosome formation (and hence an increase in autophagy initiation) and a decrease in the rate of autophagic degradation (including fusion of autophagosomes and lysosomes, and autolysosome degradation). In order to track autophagic flux, CQ was

Fig. 6. Protective role of Mfn2 in I/R was through modulation of autophagy. (a) Western blotting and quantification analysis of LC3 II in the different groups. (b) Western blot and quantification analysis of p62 in the different groups ($n = 4$). (c) Fluorescence of mRFP-GFP-LC3 was detected by confocal microscopy. (d) The number of autophagosomes or autolysosomes was counted per neuron, and quantification analysis is presented (over 200 cells were counted in each individual group; $n=3$). The data represent the mean $\pm$ SEM (two-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the untreated neurons.

used to detect LC3 II levels in both the control and I/R groups. However, it remained unclear whether autophagic degradation simultaneously decreased or not. Increased p62 expression indicated that I/R could also inhibit autophagic degradation. These results demonstrated that induced I/R increased autophagosome formation and inhibited autophagic degradation. This was in agreement with previous reports that demonstrated that I/R injury could inhibit the degradation of autophagosomes, block the autophagic flux, and promote cell injury in cardiac ischemia [34, 35]. Results from the mRFP-GFP-LC3 transfection section showed an accumulation of autolysosomes, which suggested a decrease in the degradation of autolysosomes in I/R. Previously, Zhang et al., attributed the increased number of autophagosomes/autolysosomes in I/R-treated neurons only to increased autophagosomes [26]. We speculated that this discrepancy may be ascribed to the inability to distinguish autophagosomes from autolysosomes in their experimental model. Moreover, the study by Zhang et al. depended solely on lysosomal inhibitors to monitor the autophagy reflux. Therefore, it was possible that the lysosomal inhibitors would partly influence the inhibitory effect induced by I/R. It was worth mentioning that in the present study we assessed autophagic flux via monitoring the fluorescence of mRFP-GFP-LC3 constructs. In addition, in our experiment little mitophagy were not founded by TEM, which seemed not consist with some previous researches [36, 37]. The results of TEM showed several mitochondria were swelling and vacuolar. And there was no mitochondria in the observed autophagosomes and autolysosomes. Moreover, with aid of technique of Immunoelectron Microscope (IEM), the labelled Mfn2, a marker of mitochondrial, were not founded in autophagosomes and autolysosomes by employing IEM after I/R injury (Fig. 7). In this picture, labelled Mfn2 located on the edge of autophagosome (Fig. 7, red arrow) maybe due to mitochondria supply membranes for autophagosome, and Mfn2 might be the main regulating factor [20]. By analyzing the distinction between each other, the different experiment conditions and different evaluation criterions might be the dominating reasons.

Increased autophagy was involved in neuroprotection thus acting as a compensatory protective program for I/R injury [38, 39]. Suppressed autophagy was reported to be associated with neural loss and neurobehavioral deficits in mice, eventually leading to neurodegenerative diseases [40, 41]. In contrast, Wen et al., reported that 3-MA and other autophagy inhibitors could reduce infarct volume, brain edema, and motor deficits in a permanent focal cerebral ischemia model [42]. Another study also indicated that inhibition of autophagy played a beneficial role in modulating neurological deficits after I/R observed under conditions of a lower level of estradiol [43]. In addition, it was demonstrated that 3-MA could not only attenuate cell apoptosis but also reduce migration via autophagy in I/R [44]. Therefore, the exact role of autophagy in I/R conditions is still controversial. In present

Fig. 7. The picture was captured by Immunoelectron Microscope (IEM). Autophagosomes were pointed by a black thick arrow. The black spot pointed by a black thin arrow was labelled Mfn2 which located on mitochondrial. And the labelled Mfn2 which located on autophagosome pointed by red arrow might due to Mfn2 supplied and regulated the autophagosome formation.

study, we observed that autophagy inhibitors 3-MA, CQ and E64/Pep A could aggravate I/R injury in neurons. Furthermore, the results indicated that although the effect was limited, rapamycin exerted a protective role yet after incubation in the reperfusion phase. This might be explained by the fact that rapamycin promotes autophagy at the base line, but has no effect on I/R induced autophagic degradation inhibition.

Mfn2 is attracting increasing attention due to its potential role in autophagy. Previously, we reported that Mfn2 could attenuate hypoxic injury through a mitochondria-dependent pathway [22]. The results of the present study also indicated that overexpression of Mfn2 played a protective role in I/R injury. Overexpression and knock-down results indicated that Mfn2 could promote expression of LC3 II. Hailey *et al.* reported that cells lacking Mfn2 did not form autophagosomes in response to starvation [20]. Similarly, previous studies reported an interaction between Mfn2 and RAB7, the small GTP-binding protein required for autophagy [45, 46]. Therefore, the protective role of Mfn2 in I/R might be attributed to the promotion of autophagy. In the early reperfusion phase, elevated Mfn2 levels might play a compensatory protective effect in autophagy. On the other hand, in the late reperfusion phase, the reduced Mfn2 expression demonstrated that this compensatory effect became non-functional. Additionally, with the aid of mRFP-GFP-LC3 constructs, we demonstrated that Mfn2 could promote the fusion of autophagosomes and lysosomes. According to our previous data, I/R could induce impaired autophagic degradation and the impaired autophagic degradation, which both aggravated I/R injury. The p62 results indicated that Mfn2 did not lead to a more autolysosomal accumulation after I/R injury. The results demonstrated that the protective role of Mfn2 in I/R might be through increased autophagy without increased autolysosome accumulation, indicating that Mfn2 did not restore the inhibited autophagic degradation induced by I/R. The possible role of Mfn2 in promoting autophagosomal fusion of autophagosome and lysosome indicated some potential relationship between Mfn2 and lysosome. Therefore, the exact role of Mfn2 in the lysosomal system still needs further research. In addition, as we all know that autophagy was triggered during nutrient and energy deprivation in a variety of cells as a homeostatic response to metabolic stress [47]. Moreover, in our previous study, it was demonstrated that Mfn2 could ameliorate the disrupted ATP induced by hypoxia injury. Maybe ATP play a role in the mechanism of Mfn2 and autophagy. However, the mechanism in Mfn2 and autophagy is complex, and there is no directed evidence could illustrate whether the promoted effect of Mfn2 in autophagy is through regulation of ATP levels or not. Therefore, there are several unsolved issues needed to be further research.

Conclusion

In summary, by using an *in vitro* model for studying I/R injury, we demonstrated an increase in autophagy after I/R. Additionally, our research also verified the impaired degradation of autolysosomes in I/R conditions. Moreover, results revealed that Mfn2 could ameliorate I/R injury through increasing autophagy, without increasing autolysosome accumulation induced by I/R. To the best of our knowledge, this is the first study to investigate the role of Mfn2 in I/R injury and its relationship to autophagic flux. Also, we provided a detailed description autophagic flux induced by I/R. These results may represent a potential novel strategy for neuroprotection against cerebral I/R damage.

Abbreviations

ANOVA (analysis of variance); CCK-8 (cell counting kit-8); Ct (critical threshold); CQ (Chloroquine); DMEM (Dulbecco's modified Eagle's medium); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); I/R (Ischemia/reperfusion); LV-Mfn2 (overexpression lentivirus of Mfn2); LC3 (microtubule-associated protein light chain 3); Mfn1 (2 mitofusin 1, 2); MMP

(mitochondrial membrane potential); PBS (phosphate-buffered saline); PCR (polymerase chain reaction); Pep (A pepstain A); Sh-Mfn2 (short hairpin RNA of Mfn2).

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Disclosure Statement

No conflict of interests exists.

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