Research report

Autophagy-regulated AMPAR subunit upregulation in in vitro oxygen glucose deprivation/reoxygenation-induced hippocampal injury

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Abstract

Autophagy has been implicated to mediate experimental cerebral ischemia/reperfusion-induced neuronal death; the underlying molecular mechanisms, though, are poorly understood. In this study, we investigated the role of autophagy in regulating the expression of AMPAR subunits (GluR1, GluR2, and GluR3) in oxygen glucose deprivation/reperfusion (OGD/R)-mediated injury of hippocampal neurons. Our results showed that, OGD/R-induced hippocampal neuron injury was accompanied by accumulation of autophagosomes and autolysosomes in cytoplasm alongside a dramatic increase in expression of autophagy-related genes, LC3 and Beclin 1 and increased intracellular Ca2+ levels. Pre-treatment with autophagy inhibitor 3-methyladenine (3-MA) significantly reduced this effect. Moreover, the OGD/R-induced upregulation of mRNA and protein expressions of GluR1, GluR2, and GluR3 were also effectively reversed in cells pretreated with 3-MA. Our findings indicate that OGD/R induced the expression of GluRs by activating autophagy in in vitro cultured hippocampal neurons, which could be effectively reversed by the administration of 3-MA.

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1. Introduction

Cerebral ischemia-reperfusion (I/R) injury is a pathological phenomenon that occurs after restoration of blood supply to brain tissues subsequent to ischemia or hypoxia (Carden and Granger, 2000). It typically occurs after therapeutic thrombolysis in patients with acute ischemic stroke. I/R injury aggravates neuronal insult and may lead to disability and death (Bai and Lyden, 2015; Pan et al., 2007). Treatment outcomes of I/R are typically poor; innovative approaches are required to prevent brain damage induced by I/R (Eltzschig and Eckle, 2011).

Recent studies have demonstrated a pivotal role of autophagy in the regulation of I/R-induced neuronal cell injury (Descloux et al., 2015; Gabryel et al., 2012; Xu et al., 2012, 2013; Zhang et al., 2013). Autophagy is an essential intracellular catabolic pathway responsible for the turnover of long-lived proteins and cellular components (Feng et al., 2014). Based on the mechanism of substrate-delivery to lysosome, three different forms of autophagic pathways are recognized: macroautophagy, microautophagy, and chaperone-mediated autophagy (Feng et al., 2014). Macroautophagy, hereinafter referred to as autophagy, initiates with the formation of double-membraned phagophore (also known as isolation membrane), which encapsulates intracellular materials and matures into an autophagosome (He and Klionsky, 2009). Autophagosome fuses with lysosome and degrades engulfed cargo for recycling.

Activation of autophagy in the reperfusion phase of I/R has been demonstrated in rodent brain as well as in cultured neurons following oxygen-glucose deprivation (OGD)/reperfusion (OGD/R) (Zhang et al., 2013). The autophagy inhibitor, 3-methyladenine (3-MA), has been shown to prevent death of hippocampal CA1 neurons in a rat model of I/R injury (Wang et al., 2011), which suggests that autophagic cell death may contribute to hippocampal damage induced by I/R. However, the regulatory role of autophagy in neuronal damage is yet to be fully elucidated.

Calcium overload, caused by increased Ca2+ influx from extracellular environment or release from intracellular stores, is known
to be an initial event in ischemic neuronal damage in brain (D’Orsi et al., 2015; Pringle, 2004). \( \text{Ca}^{2+} \) influx through ion channels formed by \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type (AMPA-type) glutamate receptor (AMPAR) are shown to be involved in hypoxic-ischemic brain injury (Deng et al., 2003; Gerace et al., 2015; Tang and Xing, 2013). Four subunits, namely GluR1-4, have been identified as the main components of \( \text{Ca}^{2+} \)-permeable AMPAR channels (Liu and Savtchouk, 2012). Degradation of GluR1 in hippocampal neurons has been shown to be regulated by autophagy (Shehata et al., 2012), which implies an association between autophagic pathway and GluR-modulated \( \text{Ca}^{2+} \) influx.

Hippocampus is particularly vulnerable to I/R injury (Kirino and Sano, 1984). Abundant expression of GluR1, GluR2, and GluR3 are found in hippocampal neurons (Shi et al., 2001). Based on this body of evidence, we investigated the role of autophagy in mediating OGD/R injury in cultured hippocampal neurons. The association between autophagy and AMPARs was also explored.

2. Results

2.1. OGD/R induced hippocampal neuron injury

Hippocampal neurons were subjected to OGD for 0.5, 1 or 2 h, followed by 24 h of reoxygenation. Increased neuronal damage was observed after OGD/R (Fig. 1A) CCK-8 assay revealed significantly reduced viability of cells exposed to OGD/R (\( P < 0.05 \)) (Fig. 1B). These findings suggest that OGD/R significantly induced hippocampal neuron injury. In the following experiments, 0.5 h or 1 h OGD, followed by reoxygenation for indicated time duration (0–96 h) was used for establishment of OGD/R-like neuronal damage.

2.2. OGD-induced autophagic activity

In order to understand the mechanism of OGD/R-induced hippocampal neuron injury, TEM analysis was carried out. Compared with healthy control cells, OGD/R induced obvious ultrastructural change in neurons, with autophagosomes and autolysosomes being detected in the cytoplasm of damaged neurons (Fig. 2). Some of the autophagosomes and autolysosomes engulfed with damaged intracellular organelles. mRNA and protein levels of autophagy-related genes (Atgs), including microtubule-associated protein 1 light chain 3 (MAP1 LC3, or briefly LC3) and Beclin 1 (a mammalian ortholog of the yeast Atg6), were measured. As shown in Fig. 3A and B, the mRNA levels of both LC3 and Beclin 1 were significantly elevated following 0.5 h or 1 h of OGD (\( P < 0.05 \) compared with control). Persistent upregulation of mRNA expression of genes coding for LC3 and Beclin 1 was detected immediately after OGD and until 96 h after reoxygenation. When compared to 0.5 h of OGD, prolonged OGD treatment (1 h) upregulated the mRNA expression of genes coding for LC3 and Beclin 1.

Increased protein expressions of LC3-II and Beclin 1 were also detected in cells exposed to 0.5 h or 1 h of OGD following reoxygenation (Fig. 3C-E). The baseline levels of LC3-II and Beclin 1 were relatively low in the control hippocampal neurons. However, OGD treatment rapidly elevated LC3-II and Beclin 1 expression, both of which were continually upregulated until 96 h after reoxygenation. These findings suggest activation of autophagy in hippocampal neurons after OGD/R injury. Autophagy activation was more...
Fig. 3. Expressions of LC3 and Beclin 1 in hippocampal neurons. Cells were treated with 0.5 or 1 h of OGD, followed by reoxygenation for indicated time duration (0–96 h). In the 1-h OGD + 3-MA group, cells were incubated with 5 mM of 3-MA one hour prior to OGD. Subsequently, cells were exposed to 1 h OGD followed by reoxygenation. Untreated cells were used as control. mRNA expression of LC3 (A) and Beclin 1 (B) were examined by RT-qPCR. Relative ratio of the level of mRNA to that of β-actin was calculated from three independent experiments. (C) Protein expressions of LC3-I, LC3-II, and Beclin 1 were determined by Western blotting. Relative ratio of the level of LC3-II (D) and Beclin 1 (E) to that of β-actin was calculated from three independent experiments (n = 3). Comparisons between-group differences assessed using one-way Analysis of Variance (ANOVA) and least-significant difference (LSD) test. *P < 0.05 compared with control; #P < 0.05 compared with OGD 0.5 h group. OGD, oxygen glucose deprivation; 3-MA, 3-methyladenine.

Fig. 2. Representative TEM images showing ultrastructural changes in OGD/R-treated hippocampal neurons. (A) Healthy control neurons; (B) Hippocampal neurons exposed to 1 h of OGD, followed by 24 h of reoxygenation. Ultrastructural changes of cells were examined by TEM. Scale bar, 1 μm. Damaged neurons exhibited abundant vacuoles and autolysosome. Vacuoles are indicated by black arrows, and autolysosome engulfed with materials is indicated by red arrow. TEM, transmission electron microscope; OGD/R, oxygen glucose deprivation/reperfusion.
apparent in neurons after 1 h of OGD as compared to that after 0.5 h of OGD.

2.3. 3-MA suppressed OGD-induced autophagy

Pretreatment with an autophagy inhibitor, 3-MA, significantly reduced the mRNA and protein expressions of LC3 and Beclin 1 following 1 h of OGD and reoxygenation, as revealed on RT-qPCR and Western blot analysis (Fig. 3). TEM analysis showed a decrease in number of autophagosomes and autolysosomes in cells (Fig. 4). These findings demonstrate that 3-MA inhibited autophagy in hippocampal neurons subjected to OGD/R-induced cell injury.

2.4. Effect of 3-MA on cell survival and intracellular calcium levels

The effects of autophagy inhibition on intracellular calcium levels were assessed using Fluo-3 AM probe. Cells subjected to 1 h OGD followed by 24 h reoxygenation exhibited a significantly enhanced (1.98-fold increase) fluorescent intensity as compared to that in control (P < 0.05), which indicated accumulation of intracellular Ca^{2+} level upon OGD/R injury (Fig. 5). Pretreatment with 3-MA markedly attenuated this effect (P < 0.05 vs. untreated cells). However, compared with control, the intracellular Ca^{2+} level in 3-MA + OGD/R group was still higher (1.28-fold increase).

2.5. Effect of 3-MA on GluR level

In order to understand the involvement of AMPAR subunits in OGD/R-induced neuronal injury, we assessed the expression of GluR subunits. An obvious increase in the mRNA expression of GluR1, GluR1, and GluR3 was detected within 24 h of reoxygenation (Fig. 6A, B). A significant upregulation of GluR1, GluR1, and GluR3 levels was also observed in hippocampal neurons following OGD/R. Pretreatment with 3-MA, however, remarkably attenuated this effect (Fig. 6C-E). These findings indicate that the enhanced expression of GluR subunits in hippocampal neurons subjected to OGD/R-induced cell injury may be mediated by autophagy.

3. Discussion

In this study, we observed a rapid and sustained induction of autophagy in cultured rat hippocampal neurons following OGD/R injury. Pretreatment with 3-MA significantly reduced the intracellular Ca^{2+} concentration and GluR expression in hippocampal neurons. Increased expression of autophagy-related proteins, LC3-II and Beclin 1, and reduced expression of autophagy-specific substrate p62/SQSTM 1, has been widely detected in neuronal cells following OGD/R injury (Yu et al., 2014; Zhang et al., 2013). Our results are consistent with previous reports. Compared to 0.5-h of OGD treatment, prolonged OGD treatment (1 h) exacerbated autophagic activity. Administration of 3-MA significantly abolished
autophagy induction as well as cell injury, indicating autophagy may play a deleterious role in hippocampal survival following OGD/R.

Inhibition of autophagy has been shown to alleviate neuronal damage after cerebral ischemia, both in cell culture and rodent models (Koike et al., 2008; Li et al., 2015; Wang et al., 2016; Zhang et al., 2014; Zheng et al., 2014). Therefore, blockage of autophagy is a potential target for prevention of I/R injury-induced neuronal damage.

Maintenance of cytoplasmic Ca²⁺ homeostasis is another strategy to prevent I/R injury, as abnormal influx of Ca²⁺ through Ca²⁺ channels contributes to neuronal toxicity (Tuttolomondo et al., 2009). In the current study, increased cytoplasmic Ca²⁺ concentration was detected in hippocampal neurons 24 h after OGD/R. Similar in vitro results have been reported earlier (He et al., 2014). Of note, pretreatment with 3-MA effectively attenuated the increase in cytoplasmic Ca²⁺ levels, which suggests that prolonged autophagic stress may lead to Ca²⁺ toxicity and neuronal cell death in OGD/R model.

Molecular mechanisms that mediate the interplay between autophagy and calcium homeostasis are not clear. Endoplasmic reticulum (ER) stress is thought to be a common upstream factor that modulates both Ca²⁺ levels and autophagy (Dubois et al., 2016; Su and Li, 2016). Moreover, abnormal calcium homeostasis has also been reported to alter the autophagic flux (Sasaki et al., 2015), which suggests a loop control between autophagy and calcium homeostasis, and that maintenance of both autophagic activity and intracellular calcium level at an appropriate level is important for maintenance of neuronal cell integrity.

In a previous study, Ca²⁺-permeable AMPA receptor was shown to trigger motor neuron death in sporadic amyotrophic lateral sclerosis (ALS) (Kwak et al., 2010). Consistent with this finding, neuronal damage in our study was accompanied by enhanced mRNA and protein expression of Ca²⁺-permeable AMPA receptors, including GluR1, GluR2 and GluR3. It is possible that in OGD/R treated neurons, increased GluRs level leads to excitotoxicity and subsequently results in Ca²⁺ overload. Importantly, the upregulation of GluR1, GluR2 and GluR3 caused by OGD/R was effectively reversed by autophagy inhibition, which indicates that autophagy may mediate the degradation of GluRs in hippocampal neurons.

Consistent with this hypothesis, Shehata et al. detected an autophagy-mediated GluR1 clearance in hippocampal neurons (Shehata et al., 2012). To confirm the role of autophagy in modulating GluRs and cytoplasmic Ca²⁺ homeostasis in damaged neurons, further studies are required to investigate the autophagic flux in cells exposed to OGD/R.

In this study, OGD/R induced upregulation of GluR, increased cytoplasmic Ca²⁺ levels, and reduced neuronal viability in cultured hippocampal neurons. These events appear to be regulated by enhanced autophagy. Autophagy may prove to be a potential target for prevention of hypoxic-ischemic brain injury.

4. Experimental procedures

4.1. Reagents

Neurobasal culture medium, DMEM/F12 culture medium, B27 and D-Hank’s solution were purchased from Gibco (Life Technologies, Carlsbad, CA). Poly-D-lysine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen (Life Technologies, Paisley, UK). Polyclonal anti-LC3, anti-Beclin 1, anti-GluR1, anti-GluR2, and anti-GluR3
antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti-β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies, including goat anti-mouse and goat anti-rabbit antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and MultiSciences (Lianke) Biotech (Hangzhou, China), respectively. Autophagy inhibitor 3-methyladenine (3-MA) and Fluo 3-AM were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Primary culture of rat hippocampal neurons

Newborn Sprague-Dawley (SD) rat pups (specific pathogen free [SPF] level) were provided by Shanghai SLAC Laboratory Animal Co., Ltd, China. The protocols, which include all surgical procedures and animal usage, were approved by the Animal Care and Use Committee of Soochow University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Animals were sterilized with 70% ethanol, and the head was removed from the body. The bilateral hippocampus was isolated and transferred onto a culture dish containing DMEM/F12 medium. Hippocampus was then minced into small pieces (approximately 0.5 × 0.5 × 0.5 mm³ in size). After washing with D-Hank’s solution, tissues were digested with trypsin solution for 10 min at 37 °C. After trituration, cells were dispersed, suspended in DMEM/F12 culture medium containing 10% fetal bovine serum (FBS) and seeded onto six well plates precocated with poly-D-lysine (100 μg/mL), at a density of 7 × 10⁵ cells/mL. Cell cultures were maintained in a CO₂ incubator at 37 °C. Twenty-four hours after initial seeding, culture medium was replaced with Neurobasal medium containing 2% B27 and 1% glutamine. Culture medium was partially refreshed every three days. Cultures were identified by immunocytochemistry using neuronal markers (NSE, MAP-2) and astrocyte markers (GFAP). Cultured neurons were NSE-positive, MAP-2-positive, and GFAP-negative. Ten days after seeding, mature hippocampal neurons were used for experiments.

4.3. Establishment of OGD/R model and drug interference

Cells were randomly assigned to control, OGD and OGD+3-MA groups. In order to establish OGD model, the culture medium of hippocampal neurons was changed to glucose-free Earle’s balanced salt solution (EBSS) on Day 10 of in vitro culture. Cultures were placed in a 2-L closed container filled with 50 mL/L CO₂ and 950 mL/L N₂ mixture at 37 °C to induce hypoxia. After 0.5 h, 1 h or 2 h, cells were removed from the container, replaced with fresh neurobasal culture medium, and incubated under normal conditions to achieve reoxygenation. At the indicated time point following reoxygenation, cells were used for experiments. In OGD+3-MA group, cells were pretreated with 5 mM 3-MA at 60 min before OGD as indicated previously (Wang et al., 2011).

4.4. CCK-8 assay

Cell viability was determined by cell counting kit-8 (CCK-8), according to manufacturer’s instructions (DOJINDO, Shanghai). In brief, 10 μL of CCK-8 solution was added into culture medium, and cells incubated for 2 h at 37 °C. The absorbance value was measured at 450 nm (A450) using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was determined by the following equation:

\[
\text{Cell viability} \, (\%) = \frac{\text{Absorbance value of sample}}{\text{Absorbance value of control}} \times 100\%.
\]

4.5. Transmission electron microscopy

The ultrastructural changes associated with the formation of autophagosomes were examined using transmission electron microscope (TEM). Briefly, cells were collected by digesting in 0.25% trypsin. After centrifuging at 1,000 r/min for 5 min, the supernatant was discarded and cells were washed twice with phosphate buffer solution (PBS). Cells were pre-fixed in 2.5% glutaraldehyde for 2 h and post-fixed in 1% osmic acid for another 1 h at 4 °C. After staining in 1% uranyl acetate for 2 h, samples were dehydrated in an acetone gradient (50%, 15 min; 70%, 15 min; 80%, 15 min; 90%, 15 min; 100%, 15 min; 100%, 2 × 10 min). After dehydration, samples were impregnated with a mixture of acetone/EPON812 (1:1, 2 h, 37 °C; 1:4, overnight, 37 °C; EPON812 only, 2 h, 45 °C). 50 nm thick sections were prepared and images acquired using JEM 1230 transmission electron microscope (JEOL, Japan).

4.6. Real time-quantitative PCR

mRNA expression of target genes were determined by real time quantitative PCR (RT-qPCR). In brief, hippocampal samples were triturated with 1 mL of Trizol. A total of 200 μL of chloroform was added to the sample solution to induce phase separation. After centrifugation at 13,000 rpm for 15 min, the supernatant was collected in an RNase-free tube and isopropanol added to each sample. After 10–15 min incubation on ice, samples were centrifuged again and the RNA pellet was washed twice with 75% ethanol, and dissolved in 30 μL DEPC-treated water. Equal amount of RNA was reverse transcribed into complementary DNA (cDNA), followed by PCR amplification. PCR reaction was carried out using specific forward and reverse primers targeting different genes and were synthesized by Sangon Biotech, Shanghai, China: β-actin, forward, 5’-CCCATCTATGAGGTTACC-3’; reverse, 5’-TTTAAATGTACGAGATTT-3’; beclin1, forward, 5’-TTCAAGATCCTGGACCGAGTGC-3’; reverse, 5’-AGACACCACCTCTGGCAGTTGC-3’; β3, forward, 5’-ATCACATCTGACGAGGAGG-3’; reverse, 5’-TGCTGGATCATCAACACACG-3’.

For PCR amplification, 2 μL of template cDNA was mixed with 2 μL of forward and reverse primer (1 μL for each), 10 μL of SYBR Green PCR Master Mix (2×), as well as 6 μL of deionized water. The reaction was performed using a Roche real-time PCR instrument (Switzerland). Samples were pre-denatured at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s; annealing at 60 °C for 15 s; and extension at 72 °C for 30 s.

4.7. Western blotting

The protein expressions of LC3 and Beclin 1 were measured by Western blotting. Culture medium was removed and cells were washed with precooled PBS for three times. After that, cells were lysed with lysis buffer supplemented with PMSF (1:100) (Beyotime Institute of Biotechnology, Haimen, China) for 30 min. Samples were collected and centrifuged at 12,000 rpm for 4 min at 4 °C. The supernatant was transferred to a new tube. Equal amount of protein sample was loaded and separated in 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was then transferred onto a PVDF membrane (Merck Millipore, MA, USA). Subsequently, the membrane was blocked in blocking solution containing 5% non-fat milk for 1 h. After incubation, samples were probed with primary antibodies (anti-LC3 antibody (1:2000 dilution), anti-Beclin 1 (1:2000 dilution), and anti-β-actin (1:3000 dilution), for 1 h at room temperature (RT) followed by overnight incubation at 4 °C. On the next day, membranes were washed three times with tris buffered saline plus.
TWEEN-20 (TBST) and incubated with secondary antibodies (1:4000 dilution) for 1 h at RT. After washing three times with TBST, samples were visualized by Beyo ECL Plus kit according to manufacturer’s instructions (Beyotime Institute of Biotechnology, Haimen, China). Bands were acquired and analyzed by Image Pro 6.0 imaging analysis software. The expression of target band relative to the internal control, β-actin, was calculated.

4.8. Measurement of intracellular calcium level

The intracellular calcium level was determined by fluo 3-AM staining. Briefly, cells were washed with Hank’s buffered salt solution (HBSS) for three times, and incubated with 5 μM fluo 3-AM solution diluted in HBSS. After 30 min incubation at 37 °C, staining solution was removed, and cells washed with HBSS again. HBSS containing 1% FBS was added to the cells. After 20–30 min incubation at 37 °C, the fluorescence was examined by a flow cytometry (FC500, Beckman Coulter, FL, USA) at 480–500 nm excitation wavelength and 525–530 nm emission wavelength.

4.9. Statistical analysis

Data processing and analysis was performed using SPSS16.0 (SPSS Inc., Chicago, IL, USA) software. The mean ± SD values presented in the figures were calculated from three independent experiments. Differences between groups were assessed using one-way Analysis of Variance (ANOVA) and least-significant difference (LSD) test. For paired comparison were evaluated using two-tailed Student’s t-tests. P value < 0.05 was considered as statistically significant.

Competing interests

The authors have no competing interests.

Authors’ contributions

B.L. and L.R.H. had primary responsibility for study design, data analysis and manuscript preparation. L.M. participated in the establishment of OGD model and prepared the draft manuscript. JMF and L.G. helped with model establishment and conducted experiments involving electron microscopy. H.X. participated in establishment of OGD model. Y.Y.Y. participated in q-PCR analysis. B.S. helped in data analysis. X.L.X. and F.X. contributed to the study establishment of OGD model. Y.Y.Y. participated in q-PCR analysis. L.M. participated in the data processing and analysis using SPSS16.0 (SPSS Inc., Chicago, IL, USA) software. The mean ± SD values presented in the figures were calculated from three independent experiments. Differences between groups were assessed using one-way Analysis of Variance (ANOVA) and least-significant difference (LSD) test. For paired comparison were evaluated using two-tailed Student’s t-tests. P value < 0.05 was considered as statistically significant.

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