Autophagy Induced by Oxygen-Glucose Deprivation Mediates the Injury to the Neurovascular Unit

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Background: Autophagy is characterized by the degradation of cellular components in autophagosomes. It plays a significant role in cerebral ischemic injury and has a complex functional connection with apoptosis. The neurovascular unit (NVU) is a structural and functional unit of the nervous system presented as a therapeutic target of stroke. This study aimed to investigate the effect of autophagy induced by ischemic damage on NVUs.

Material/Methods: SH-SYSY cells, C6 cells, and rat brain microvascular endothelial cells were cultured with oxygen-glucose deprivation (OGD) exposure for different time durations, and 3-methyladenine (3-MA) was added as an autophagy inhibitor. In all 3 cell lines, lactate dehydrogenase (LDH) release was measured. Furthermore, apoptosis was detected using Annexin V-fluorescein isothiocyanate/propidium iodide labeling and immunofluorescence staining. Autophagosomes were observed through AO/MDC (acridine orange/monodansycadaverine) double staining. LC3-II expression levels were evaluated by western blot analysis.

Results: In the OGD groups of 3 cell lines, LDH leakage, and apoptotic rates were obviously increased. Remarkable increase in LC3-II expression was found in the OGD groups of SH-SYSY cells and C6 cells. However, 3-MA decreased the LC3-II expression to varying degrees.

Conclusions: OGD could induce the over-activation of autophagy and augment the apoptotic activity in neurons and glial cells of NVUs.

MeSH Keywords: Autophagy • Hypoxia-Ischemia, Brain • Stroke

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Background

Ischemic stroke results from a sudden disruption of blood flow to the brain, leading to brain cell death and neurologic impairment. Early investigations aimed at the pathological mechanism of acute cerebral infarction merely concluded that the obstacle between the matrix and the neuron and the reduction of ATP led to the depolarization and necrosis of hypoxic neurons. With the development of advanced investigative methods, recent studies have emphasized the importance of changes in programmed cell death [1]. Although all the aforementioned aspects have focused on neurons, explicit connections have also been found between astrocytes, microglia, and vascular endothelial cells as they sustain neurons, conduct complex and specific signals, and execute ischemic cascade reaction collectively after focal cerebral ischemia. Considering the complex mechanism involved in the pathological process of ischemic stroke, drugs only acting on a single target might not be sufficient to treat ischemic stroke [2].

Since 2002, the neurovascular unit (NVU) consisting of neurons, astrocytes, smooth muscle cells, endothelial cells, pericytes, basement membrane, and extracellular matrix has been proposed as a structural and functional unit of the nervous system, which serves as part of an integrated model for treating stroke. All these components of the NVU play a dynamic role in pathological processes of acute cerebral infarction [3]. Currently, however, it is thought that it is not only neurons damaged from a stroke, but gliocytes and microvasculature that are involved, hence, protection for the NVU becomes the most important, potential strategy to address brain stroke. As it is well known, neurons, astrocytes, and brain microvascular endothelial cells (BMECs) are the key components of NVUs. A few studies have established a triple primary cell co-culture model with neurons, astrocytes, and rat BMECs as a model for NVUs in vitro [4,5], but the aforementioned studies had complex operational aspects that were difficult to culture. Every kind of cell in the NVU has its specific and essential role in physiology, pathology, and even response to drugs. Thus, we had to separately cultured 3 types of cells followed by oxygen-glucose deprivation (OGD), and examine the common injury mechanism of these 3 major cell types related to NVUs.

Looking at the neural damage after stroke, injured neurons, gliocytes, and microvessels can spread harmful effects to nearby cells via cellular interactions [6,7]. Induced by stroke, a series of pathologies may occur as dysfunctional energy metabolism, excitatory amino acids, inflammation, oxidative stress, autophagy, and apoptosis hit the neural cells [2,6]. Autophagy is considered a double-edged sword. Autophagy, triggered by mild physiological and pathological stimulation, is neuroprotective, whereas over-activation of autophagy leads to a series of detrimental consequences to neuronal survival [8]. Autophagy has been considered a significant process that may be a key regulator of ischemic injury. It has also been distinguished as the third mechanism of cell death after apoptosis and necrosis [9]. When mild ischemia occurs, moderate activation of autophagy, as an important approach of autolous repair, may remove damaged organelles, clean abnormal proteins, prevent aggregation of protein, and inhibit apoptotic cell death. After severe ischemia, continued and excessive autophagy leads to cell death directly and also interacts with the apoptosis signal [10,11]. However, the impact of autophagy induced by cerebral ischemia on NVUs is unknown. Microtubule-associated protein (LC3) is widely used to illustrate the formation and number of autophagosomes. Cysteinyl aspartate-specific protease-3 (caspase-3) is the approved biomarker of apoptosis.

Using SH-SY5Y cells, C6 cells, and RBMECs, this study investigated autophagy mediated by OGD in the NVU.

Material and Methods

Three kinds of cerebral cells

The human neuroblastoma cell line SH-SY5Y was purchased from the American Type Culture Collection (VA, USA) and was grown in RPMI 1640 Medium (Hyclone, Thermo Fisher Scientific, MA, USA) with 10% fetal bovine serum (FBS; Hyclone). The cells were incubated in a 5%/95% mixture of CO2 and atmospheric air with humidity at 37°C. One day after plating, cells were incubated in neurobasal medium, supplemented with 2% B27 (Gibco) and 0.5 mM L-glutamine (Gibco, 35050-061, USA). The cells were induced to differentiate into a homogeneous population of cells with neuronal morphological structure by adding 10 M retinoic acid (Sigma, USA) to the medium for 3 days [12,13]. The cells were then used in our experiments.

Rat C6 glioma cell line was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China, No. 3111C0001CCC000131). C6 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) with 5% FBS (Thermo, Waltham, MA, USA) at 37°C. When cell growth approached 90%, cells were digested with 0.25% trypsin followed by cell passage. Cells within 6 passages were used in this experiment.

RBMECs were purchased from American ScienCell Research Laboratories (Carlsbad, CA, USA). Cells from passage 6 to passage 8 were cultured in DMEM medium with 10% FBS, 20 μg/mL bFGF, and 100 μL/mL heparin under standard conditions [14,15].

The NVU model was established consisting of SH-SY5Y cells, C6 cells, and RBMECs.
OGD induction and treatment

In the OGD groups, the original culture medium was removed, and then the cells were washed with Krebs medium. Then the cells, with medium, were placed in a humidified incubator with 95% N₂ and 5% CO₂ at 37°C for 5 minutes. The incubator was then sealed, and the cells incubated at 37°C for 10 minutes. During the experimental process aforementioned, O₂ concentration was kept at 0.3–1.0%, monitored by an O₂ analyzer. At the end of the OGD period, instead of OGD medium, basal medium with glucose was used to wash the cells and then added again. The cells were then placed in an incubator with normal oxygen content for reoxygenation.

In the control groups, all treatments were the same as those in the OGD groups except the cells were not treated with OGD or the reoxygenation process. For the 3-MA groups, one additional treatment was given: the cells were treated with a solution of 30 mmol/L 3-MA that had been prepared in normal basal medium. The final concentration of the 3-MA was 1 mmol/L.

MTT [3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide] assay

For the detection of cell viability, MTT [3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide, v13154, Gibco] assay was performed using a final concentration of MTT of 2.5 mg/mL, and incubation for 4 hours. Additionally, 150 µL of dimethyl sulfoxide (DMSO; Sigma, MO, USA) was added to each well. A microplate reader at 570 nm was used to read the optical density (OD).

Lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase (LDH) activity was measured using the LDH cytotoxicity detection kit (3004732122, Roche). The supernatant (100 µL) and reaction mix (100 µL) were mixed in 96-well plates and incubated for 0.5 hours at room temperature. The absorbance of all samples was measured applying a microplate reader at 490 nm.

Annexin V-fluorescein isothiocyanate/propidium iodide staining

Applying the detection kit (APOAF, Sigma), Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining were used to observe apoptotic cells. After washing with phosphate-buffered saline (PBS), experimental cells were resuspended in binding buffer. Then, 5 µL of Annexin V-FITC and 5 µL of PI (50 µg/mL) were added to the binding buffer for 5 minutes in the dark. The samples were immediately measured using a flow cytometer. Apoptotic cells were expressed as a percentage of the total number of cells stained. All experiments were performed in triplicate and repeated twice to assess the consistency of response.

AO/MDC double staining

The cells were washed with 1x PBS, twice, then incubated for 15 minutes at room temperature for AO (9231, Sigma, 1 µg/mL) staining. Then, the cells were washed with 1x PBS twice, observed, and photographed under a fluorescence microscope. For MDC staining, MDC (D4008, Sigma, 50 µM) was incubated for 1 hour at 37°C. The cells were then washed with 1x PBS, twice. A fluorescence microscope (Olympus/BX51) was used to photograph the cells.

Immunofluorescence staining

The cells were fixed in 4% polyformaldehyde for 5 minutes, then washed with 1x PBS and 0.25% Tween 20 (PBST), 3 times. After that, the cells were blocked with 5% goat serum at room temperature for 30 minutes. The following primary antibody was incubated at 4°C overnight: cleaved caspase-3 rabbit monoclonal antibody (9664, Cell Signaling). After washing again, the cells were transferred to incubate with the fluorescent secondary antibody immunoglobulin (F0382, anti-rabbit Alexa Fluor 594-conjugated, 1: 100, Sigma) at 37°C for 1 hour [16]. Subsequently, the cells were rinsed with PBS and incubated with DAPI (4',6-diamidino-2-phenylindole) solution (1: 200) at room temperature for 15 minutes, then the sections were photographed by a fluorescence microscope.

Western blot analysis

Protein extracts were isolated from each group of cells using RIPA protein lysis buffer containing 1 mM PMSF (phenylmethylsulfonyl fluoride). As the standard, protein concentration was determined using bicinchoninic acid (BCA, 23225, Thermo). Equal amounts of protein were loaded on 10% polyacrylamide gels (14100312, Novex) and transferred onto the polyvinylidene difluoride membrane. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS). Then membranes were incubated in 1: 1000 dilution of rabbit LC3-II polyclonal antibody (3868, CST) and 1: 200 dilution of mouse anti β-actin monoclonal antibody overnight at 4°C. The blots were then incubated with secondary antibodies (1: 2000, Jackson Immuno Research). Finally, the ECL (electrochemiluminescence) developer was added, and the protein electrophoresis bands were photographed. Using the ImageJ software, the OD of objective protein electrophoresis bands was analyzed.

Statistical analysis

All quantitative data were presented as mean ±SEM (standard error of the mean). One-way analysis of variance (ANOVA) with
Bonferroni/Dunnett post-hoc tests were performed for multiple comparisons. A $P$ value less than 0.05 was considered statistically significant.

**Results**

**Impact of different OGD times on SH-SY5Y cells, C6 cells, and RBMECs**

Compared with the control group at each time point, the OD value of the 3 cell types (SH-SY5Y cells, C6 cells, and RBMECs) in OGD groups (0-, 3-, 6-, 12, and 24-hours of OGD) decreased at 6–24 hours after OGD administration. As shown in Figure 1, the appropriate OGD time for SH-SY5Y cells, C6 cells, and RBMECs was 12 hours (Figure 1A), 24 hours (Figure 1B), and 12 hours (Figure 1C), respectively. Hence, these groups were chosen as our experimental OGD groups.

Compared with the control group at each time point, the OD of SH-SY5Y cells in the OGD 12 hours/reoxygenation 6 hours group (OGD12h/R6h group) and the OGD12h/R12h group, and the C6 cells in the OGD24h/R2–24h group, and the RBMECs in the OGD12h/R6–24h group had significantly reduced OD values ($P<0.01$).

These results showed that the appropriate time of cellular damage in SH-SY5Y cells, C6 cells, and RBMECs were OGD12h/R12h, OGD24h/R6h, and OGD12h/R6h (Figure 1D), respectively.
Autophagy induced by OGD mediates the injury of NVU

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OGD-induced damage regulated LDH release from SH-SY5Y cells, C6 cells, and RBMECs

Compared with the control groups, LDH release in the OGD groups and 3-MA groups were upregulated with significant differences (P<0.01). In contrast to the OGD groups, LDH release in the 3-MA groups decreased significantly (P<0.01), and was higher than those of the control groups. This phenomenon appeared in all 3 types of cells (Figure 2).

OGD-induced damage affected apoptosis of SH-SY5Y cells, C6 cells, and RBMECs

For SH-SY5Y cells, compared with the control group, the OGD group revealed activated caspase-puncta by being stained with enhanced green fluorescence (Figure 3A) and showed an obviously increased apoptotic rate with a statistically significant difference (P<0.01) (Figure 3B, 3C). Compared with the OGD group, the 3-MA group was stained with weakened green fluorescence (Figure 3A). The 3-MA group showed a remarkable reduced apoptotic rate with a statistically significant difference than that in the OGD group (P<0.01), which was still obviously higher than the control group (Figure 3B, 3C).

For C6 cells, in contrast to the control group, the OGD group was stained with enhanced green fluorescence (Figure 3D) and showed an obviously higher apoptotic rate with a statistically significant difference (P<0.01) (Figure 3E, 3F). Meanwhile, the nucleus in the OGD group stained with blue fluorescence presented a state of contraction and dispersion, which was different from that found in the control group (Figure 3D). Compared with the OGD group, the 3-MA group was stained with weakened green fluorescence (Figure 3D) and showed an obviously lower apoptotic rate with a statistically significant difference (P<0.01), which was still obviously higher than the control group (P<0.01) (Figure 3E, 3F).

For RBMECs, no fluorescence staining of activated caspase-3 was observed, while the apoptotic rate increased significantly in the OGD group (P<0.01) (Figure 3G, 3H). In contrast to the OGD group, the 3-MA group showed a decreased apoptotic rate, which was still higher than the control group (P<0.05) (Figure 3G, 3H).

OGD-induced damage affected autophagy of SH-SY5Y cells, C6 cells, and RBMECs

For SH-SY5Y cells, almost no autophagosomes appeared and the expression level of LC3-II was low in the control group and 3-MA group, with no statistically significant difference between these 2 groups (Figure 4A). Compared with the control group, a large number of autophagosomes (shown as red signals in AO staining and enhanced green signals in MDC staining) were observed, and the expression level of LC3-II increased with a statistically significant difference (P<0.01) in the OGD group (Figure 4B, 4C).

For C6 cells, almost no autophagosomes appeared in the control group or the 3-MA group (Figure 4D). The expression level of LC3-II was slightly higher in the 3-MA group than in the control group (P<0.05) (Figure 4E, 4F), but both were low. Compared with the control group, autophagosomes were observed, and the expression level of LC3-II augmented with a statistically significant difference in the OGD group (P<0.01) (Figure 4E, 4F).

For RBMECs, the expression of LC3-II was not detected. Compared with the control group, autophagosomes were found in the OGD group. The number of autophagosomes decreased in the 3-MA group in contrast to the OGD group (Figure 4G).

Discussion

Intercellular interaction is of great significance in the physiological and pathological processes of NVUs. The cells and tissues of NVUs are structurally inseparable and interconnected, resulting in immediate interaction between neuronal cells [7,17]. However, after acute cerebral infarction, the intercellular interaction between cells of the NVU may not only transmit harmful cytokines but also serve as a protective factor [9]. Therefore, each member of NVU has a specific and different role in the physiology, pathological processes, and even drug reactions. Previous studies showed a key role of endothelial cells and astrocytes in the physiopathology of neurons [18,19].

Figure 2. LDH of SH-SY5Y cells, C6 cells, and RBMECs suffered OGD stress. The LDH assay showed that OGD enhanced the cytotoxicity of SH-SY5Y cells, C6 cells, and RBMECs, whereas 3-MA decreased it (n=8 per group). * P<0.01 versus our control group, ** P<0.01 versus our OGD group. LDH – lactate dehydrogenase; RBMECs – brain microvascular endothelial cells; OGD – oxygen-glucose deprivation; 3-MA – 3-methyladenine.

Figure 3. Apoptotic rate of SH-SY5Y cells, C6 cells, and RBMECs. Compared with the control group, the OGD group showed an obviously higher apoptotic rate with a statistically significant difference (P<0.01), which was still obviously higher than the control group (P<0.01) (Figure 3E, 3F). For RBMECs, no fluorescence staining of activated caspase-3 was observed, while the apoptotic rate increased significantly in the OGD group (P<0.01) (Figure 3G, 3H). In contrast to the OGD group, the 3-MA group showed a decreased apoptotic rate, which was still higher than the control group (P<0.05) (Figure 3G, 3H).
Figure 3. 3-MA influenced apoptosis of SH-SY5Y cells, C6 cells, and RBMECs suffered OGD stress. (A) The immunochemistry analysis showed that 3-MA decline the number of activated caspase-puncta (shown as green signals) in SH-SY5Y cells. (B, C) The work of flow cytometry showed that 3-MA weakened the apoptosis of SH-SY5Y cells. (D) The immunochemistry analysis showed that 3-MA decreased the number of activated caspase-puncta in C6 cells. (E, F) Flow cytometry showed that 3-MA decreased the apoptosis of C6 cells. (G, H) Flow cytometry showed that 3-MA decreased the apoptosis of RBMECs. Scale bar: 50 μm (n=3 per group). * P<0.05, ** P<0.01 versus our control group; ## P<0.01 versus our OGD group. 3-MA – 3-methyladenine; RBMECs – brain microvascular endothelial cells; OGD – oxygen-glucose deprivation.
Autophagy, also known as type II cell death, is defined as programmed cell death along with apoptosis. The functional connection between autophagy and apoptosis is complex, which means that both may be triggered by common upstream signals (Ca²⁺, peroxide, etc.) and activated at the same time or individually under stress conditions [20]. An autophagic process is characterized by the packaging of aging cells, damaged organelles, and intracellular foreign matters in autophagosomes, followed by their transportation to lysosomes for degradation [21,22]. It is still equivocal whether autophagy is protective or fatal in ischemic NVUs. An increase in the number of autophagosomes may be a result of a more inefficient elimination of autophagosomes caused by a decrease in lysosomal activity or a blockage in autophagosome-lysosome fusion; therefore, it may not be enough to report enhanced activation of autophagy [23].

LC3 is distributed diffusely in the cytoplasm under physiological conditions (LC3-I) [24] and modified post-translationally to become LC3-II when autophagy is activated [25,26]. LC3-II constitutes the autophagosome membrane as an acknowledged biomarker of autophagy. In a study by Wang et al. [9], 3-MA reduced the OGD-induced expression level of LC3-II, intensifying LDH release and OGD-induced injury 2 hours after OGD. Furthermore, an autophagy activator (rapamycin) was used to interfere with neurons exposed to OGD after 0.5 hours. The results showed increased autophagy and enhanced neuronal activity, suggesting that the activation of autophagy was beneficial to neuronal viability in the early stage of ischemia. The present study found that in the 3 types of cells, a series of opposite consequences might be associated with OGD (6–12 hours), indicating that the cells suffered a more serious attack. The results demonstrated that OGD-induced autophagy was injurious to the NVU, whereas autophagy inhibitor 3-MA partially attenuated this damage.

LDH leakage occurs during the process of apoptosis [27,28]. With the use of autophagic inhibitor 3-MA with MTT assay, LDH leakage has been considered an indication of OGD-induced neuronal death [29,30]. As a core effective enzyme in
Figure 4. Autophagy of SH-SY5Y cells, C6 cells, and RBMECs suffered OGD stress. (A, D, G) AO and MDC immunofluorescence staining showed that 3-MA decreased the number of autophagy-puncta in SH-SY5Y cells, C6 cells, and RBMECs (shown as red signals in AO staining and enhanced green signals in MDC staining). (B, C, E, F) The Western blot analysis showed that 3-MA decreased the expression level of LC3-II in SH-SY5Y cells and C6 cells. As shown in (F), the expression level of LC3-II increased significantly in OGD group than in our control group, while 3-MA obviously decreased the expression, which was still higher than that in our control group. Scale bar: 100 μm. * P<0.05, ** P<0.01 versus our control group; ## P<0.01 versus our OGD group (n=3 per group). RBMECs – brain microvascular endothelial cells; OGD – oxygen-glucose deprivation; AO – acridine orange; MDC – monodansycadaverine, 3-MA – 3-methyladenine.
the cellular apoptotic process, cysteinyl aspartate-specific protease-3 (caspase-3) is the main executor of apoptosis, and its expression was bound to be a representation of apoptosis [31]. Carloni et al. found that rapamycin motivated autophagy and decreased the expression level of activated caspase-3 [32], the number of apoptotic cells, and cerebral injured volume in rats with cerebral hypoxic–ischemic injury. Interestingly, the data for SH-SYSY and C6 cells has indicated that OGD-induced injury augmented the activation of autophagy by increasing the expression level of LC3-II and also enhanced green fluorescence of activated caspase-3 and apoptotic rate. The differences between the study by Carloni et al. and our present study might be due to the pharmacological effects of rapamycin used to modulate autophagy-induced changes in the complex cross-talk between autophagy and apoptosis. Moreover, the models investigated in our present study and the study by Carloni et al. were not the same. Conversely, Puyal et al. reported that autophagy was detrimental to ischemic neurons [23]. They discovered that LC3 II and caspase-3 levels increased evidently after cerebral ischemia in rats with middle cerebral artery occlusion (MCAO). The expression of caspase-3 and caspase-9 and the appearance of autolysosomes were also detected. Furthermore, an intracerebroventricular injection of 3-MA reduced the lesion volume prominently. Obvious decreases in LC3-II and caspase-3 levels and increased number of caspase-9 positive cells were also found. Moreover, the translocation of apoptosis-inducing factor to the nucleus was prevented by 3-MA, indicating its neuroprotective effect against apoptosis caused by 3-MA. Qin et al. found that astrocyte viability decreased notably and LDH leakage significantly increased 6 hours after OGD [33]. Meanwhile, the fusion of autophagic vacuoles from autophagosomes and lysosomes was observed. In addition, after OGD, the protein expression level of LC3-II was markedly elevated. In contrast, 3-MA restrained the expression of LC3-II and attenuated the leakage of LDH. Furthermore, 6 hours after OGD, the expression level of Bcl-2 (an important anti-apoptotic protein) in astrocytes was evidently reduced. In addition, 3-MA significantly recovered the expression level of Bcl-2, which partly represented that 3-MA resisted OGD-induced apoptotic injury and strongly supported the findings of our present study.

In our study, conspicuously augmented leakage of LDH, labeling of caspase-3, and a higher apoptotic rate were observed in SH-SYSY and C6 cells exposed to OGD. Meanwhile, a large number of autophagosomes were detected, and the protein expression level of LC3-II increased markedly in these cells. All these OGD-induced changes were attenuated significantly in the 3-MA group. These phenomena suggest that the expression level of LC3-II protein and the apoptotic rate were upregulated in neurons and glial cells that suffered an OGD-induced injury, which is consistent with other previous empirical conclusions: the activation of autophagy acted as the promoter of apoptotic cell death and thus escalated cell death, while 3-MA inhibited autophagy and apoptosis [34,35]. In RBMECs, no expression of LC3-II protein was observed. OGD merely increased the number of autophagosomes and also the apoptotic rate. Based on these findings, we speculate that the increase in the apoptotic rate was related to the molecular mechanism of action of autophagosomes, rather than to autophagic pattern.

Conclusions

In conclusion, this present study indicated that OGD remarkably damaged SH-SYSY cells, C6 cells, and RBMECs; increased the autophagic and apoptotic rates of SH-SYSY cells and C6 cells; and increased the apoptotic rate of RBMECs. Underlying mechanisms were possibly associated with OGD-induced over-activation of autophagy and augmented apoptotic activity in neurons and glial cells.

Conflicts of interest

None.

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