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Role of Autophagy Mediated by AMPK/DDiT4/mTOR Axis in HT22 Cells Under Oxygen and Glucose Deprivation/Reoxygenation

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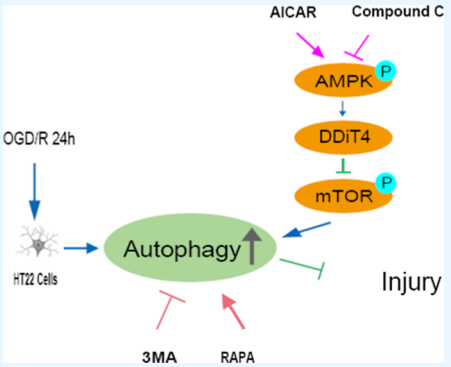
ABSTRACT: Background: cerebral ischemia/reperfusion (I/R) injury is an important complication of ischemic stroke, and autophagy is one of the mechanisms of it. In this study, we aimed to determine the role and mechanism of autophagy in cerebral I/R injury. **Methods**: the oxygen and glucose deprivation/reoxygenation (OGD/R) method was used to model cerebral I/R injury in HT22 cells. CCK-8 and LDH were conducted to detect viability and damage of the cells, respectively. Apoptosis was measured by flow cytometry and Tunel staining. Autophagic vesicles of HT22 cells were assessed by transmission electron microscopy. Western blotting analysis was used to examine the protein expression involving AMPK/DDiT4/mTOR axis and autophagy-related proteins. 3-Methyladenine and rapamycin were, respectively, used to inhibit and activate autophagy, compound C and AICAR acted as AMPK inhibitor and activator, respectively, and were used to control the starting link of AMPK/DDiT4/mTOR axis. **Results:** autophagy was activated in HT22 cells after OGD/R was characterized by an increased number of autophagic vesicles, the expression of Beclin1 and LC3II/LC3I, and a decrease in the expression of P62. Rapamycin could increase the viability, reduce LDH leakage rate, and alleviate cell apoptosis in OGD/R cells by activating autophagy. 3-Methyladenine played an opposite role to rapamycin in OGD/R cells. The expression of DDiT4 and the ratio of *p*-AMPK/AMPK were increased after OGD/R in HT22 cells. While the ratio of *p*-mTOR/mTOR was reduced by OGD/R, AICAR effectively increased the number of autophagic vesicles, improved viability, reduced LDH leakage rate, and alleviated apoptosis in HT22 cells which suffered OGD/R. However, the effects of compound C in OGD/R HT22 cells were opposite to that of AICAR. **Conclusions:** autophagy is activated after OGD/R; autophagy activator rapamycin significantly enhanced the protective effect of autophagy on cells of OGD/R. AMPK/DDiT4/mTOR axis is an important pathway to activate autophagy, and AMPK/DDiT4/mTOR-mediated autophagy significantly alleviates cell damage caused by OGD/R.



INTRODUCTION

Stroke is a leading cause of mortality and severe disability across the world, and an urgent need exists to develop new treatment.[1,2] Ischemic stroke, accounting for 60−80% of all strokes, is caused by insufficient supply of blood in the brain.[3,4] The therapeutic strategy of ischemic stroke is based on rapid vessel recanalization.[5] However, many studies have demonstrated that oxidative stress, inflammation, excitotoxicity, and apoptosis are exacerbated due to vessel recanalization.[6] The above process is so-called reperfusion injury.[7] The mechanism underlying cerebral ischemia/reperfusion (I/R) injury remains unclear, and effective therapies for cerebral I/R injury have not been established yet.

Autophagy is an essential mechanism for maintaining cell function in the CNS.[8] A large number of studies have revealed that autophagy plays a key role in cerebral I/R injury, and impairment of autophagic regulation has been observed in it.[9,10] Autophagy, an evolutionarily conserve mechanism, can degrade damaged proteins and synthesize new proteins that achieve the metabolism of the cells and the renewal of organelles through the lysosomal system; it is also an important defense mechanism against stress.[11,12] Various mechanisms such as oxidative stress and inflammation are activated by cerebral I/R injury and lead to a large amount of foreign bodies in the brain.[13] The increase of damaged cells in brain tissue leads to the occurrence of autophagy during and after I/R injury and moderating autophagy protects cells by removing damaged tissues and proteins. In summary, it seems that the induction of the autophagy process may become a potential therapeutic strategy on cerebral I/R injury, but the detailed molecular mechanisms of it remain to be addressed.

It has been shown that modulating the level of autophagy by targeting specific regulatory molecules in the autophagy machinery may impact disease progression.[14] The complicated

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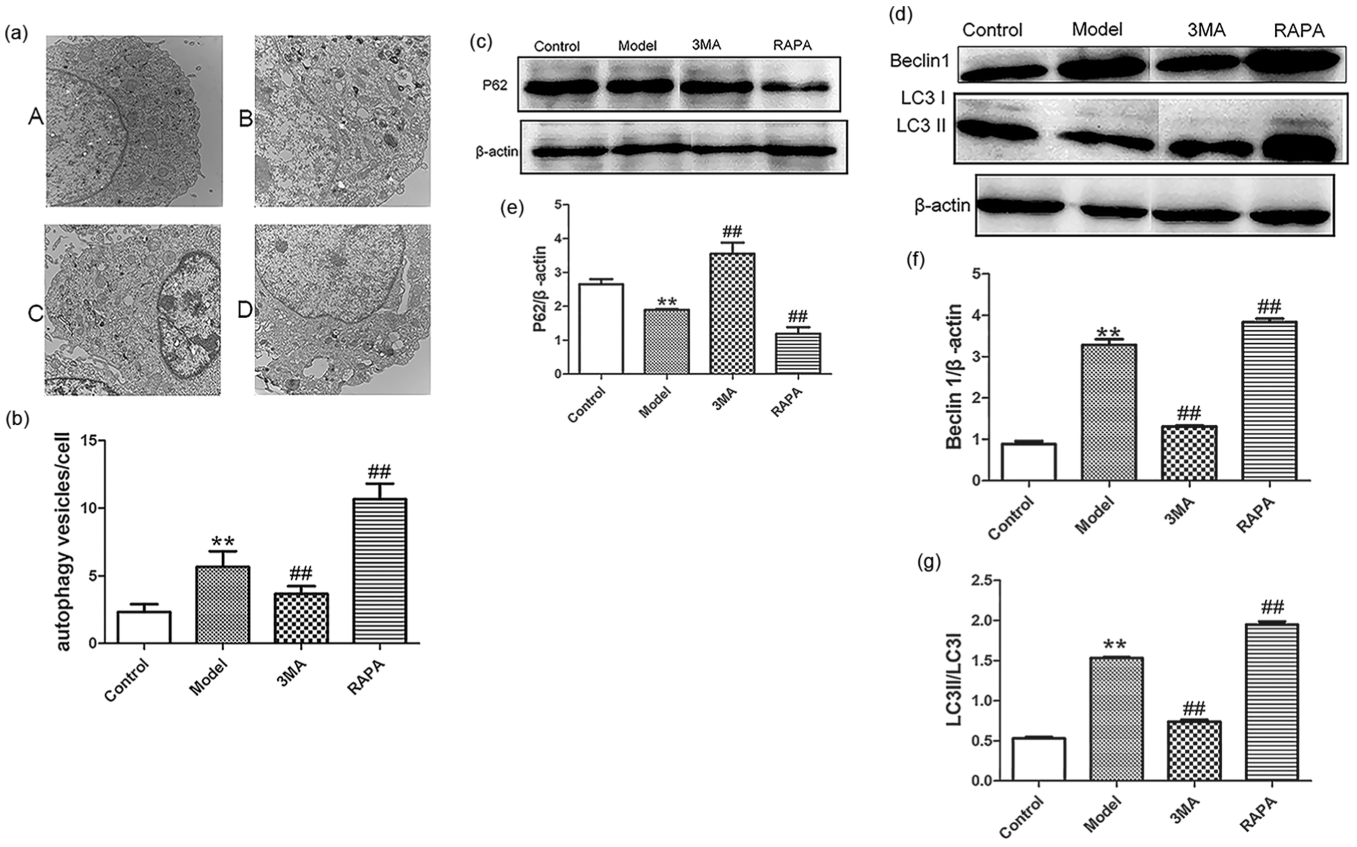

Figure 1. Level of autophagy in HT22 cells after OGD/R injury (a,b) autophagic vacuoles were observed by transmission electron microscopy; (A) control; (B) model; (C) 3MA; (D) Rapa; (c) Western blot images showed the expression levels of P62; (d) Western blot images showed the expression levels of Beclin1, LC3I, and LC3II; (e) ImageJ software was used to quantify of P62; (f) ImageJ software was used to quantify Beclin1; (g) ImageJ software was used to quantify of LC3II/LC3I (means ± SD, $^{**}P < 0.01$ vs control, $^{\#}P < 0.05$ vs model, and $^{\#\#}P < 0.01$ vs model).

interaction between multiple signaling molecules involved in the processes includes formation of phagophores, autophagosomes, and autolysosomes. In this regard, mammalian target of rapamycin (mTOR) is the "active switch" for autophagy, and numerous studies have shown that the mTOR pathway is involved regulating synaptic plasticity, neuronal transmission, axon outgrowth, and neuronal size.[15] AMP-activated protein kinase (AMPK), an important "energy susceptor" in eukaryotic cells, plays a key role in autophagy induction in response to various cellular stresses, including glucose starvation and hypoxia, and stimulates autophagy by inhibiting mTOR complex 1 (mTORC1).[16] DNA-damage-inducible transcript 4 (DDiT4) is an inhibitor of mTORC1 and can be enhanced by AMPK. DDiT4 can dissociate the TSC2/14-3-3 complex, and then AMPK inhibits mTORC1 by activating depolymerized TSC2. It is likely that the AMPK/DDiT4/mTOR axis may play an important role in the activation of autophagy. However, whether regulating the level of AMPK/DDiT4/mTOR axis has a protective effect on neurons in I/R injury remains unknown.

In this study, the OGD/R injury model of HT22 cells was used to simulate ischemia, hypoxia, and reperfusion injury in vivo. Then, the role of autophagy in OGD/R injury and the molecular mechanisms of it were explored.

RESULTS

Level of Autophagy in HT22 Cells after OGD/R Injury. Changes of autophagy in HT22 cells after OGD/R were observed. Autophagy vesicles were observed by transmission electron microscopy. The number of autophagy vesicles in the model group was increased significantly ($P < 0.01$) and was increased further in the Rapa group ($P < 0.01$), while the number of autophagy vesicles in the 3MA group was decreased significantly ($P < 0.01$) (Figure 1a,b).

Western blotting analysis showed that the level of P62 in HT22 cells was decreased significantly after OGD/R and decreased further in OGD/R HT22 cells after treatment with autophagy activator-RAPA ($P < 0.01$), but the expression of P62 in autophagy inhibitor-3MA group increased significantly ($P < 0.01$) (Figure 1c,e). The expression of Beclin1 in the model group was significantly increased, and RAPA increased the expression of Beclin1 further ($P < 0.01$), but the expression of Beclin1 in 3MA group was decreased significantly ($P < 0.01$) (Figure 1d,f). The conversion of LC3I to LC3II indicates autophagy activity, and LC3II/LC3I ratio showed that OGD/R led to a profound increase in LC3II/LC3I, autophagy activator-RAPA further improved LC3II/LC3I ratio ($P < 0.01$), but autophagy inhibitor 3MA significantly decreased LC3II/LC3I ($P < 0.01$) (Figure 1d,g). All of the above results indicated that autophagy was activated by OGD/R injury.

Effect of Regulating Autophagy on the Cell Viability after OGD/R Injury. The CCK-8 method was used to measure the cell viability after OGD/R injury in HT22 cells. The viability of HT22 cells was significantly reduced after OGD/R ($P < 0.01$) and further reduced after treatment with autophagy inhibitor-3MA in HT22 cells ($P < 0.01$). However, the viability of OGD/R HT22 cells was significantly improved after treatment with autophagy activator-RAPA compared with

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model group ($P < 0.01$) (Figure 2). The result indicated that activating autophagy is beneficial to promote the survival of cells after OGD/R.

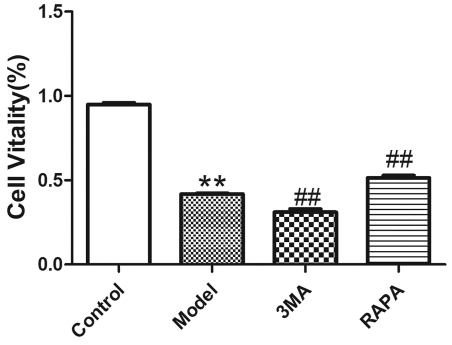

Figure 2. Effect of regulating autophagy on the viability of HT22 cells after OGD/R injury (data expressed as means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

Effects of Regulating Autophagy on the Levels of LDH in HT22 Cells Subjected to OGD/R. The level of LDH in HT22 cells subjected to OGD/R was significantly improved ($P < 0.01$), and the level of it in HT22 cells was improved further after treatment with 3MA ($P < 0.01$). However, the level of LDH decreased significantly in OGD/R HT22 cells after treatment with RAPA ($P < 0.01$) (Figure 3). The above result indicated that activating autophagy is beneficial to relieve injury of cells after OGD/R.

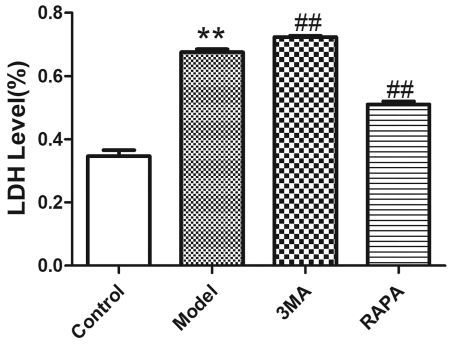

Figure 3. Effect of regulating autophagy on the levels of LDH in HT22 cells subjected to OGD/R (data expressed as means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

Effects of Regulating Autophagy on the Apoptosis in HT22 Cells Subjected to OGD/R. Apoptosis is an important mechanism of secondary damage in brain tissue after cerebral I/R injury, and there is a close relationship between apoptosis and autophagy. Flow cytometry assay was used to observe cell apoptosis after OGD/R. Flow cytometry assay showed that apoptosis rate in HT22 cells increased significantly after OGD/R ($P < 0.01$). RAPA reduced apoptosis rate in HT22 cells subjected to OGD/R by activating of autophagy ($P < 0.01$). Autophagy inhibitor-3MA increased apoptosis rate significantly ($P < 0.01$) (Figure 4).

Level of AMPK/DDiT4/mTOR Axis in HT22 Cells after OGD/R Injury. We explored the specific pathways which regulates autophagy. Western blot showed that *p*-AMPK/AMPK was increased significantly in the model group, and AICAR could further increase *p*-AMPK/AMPK, while

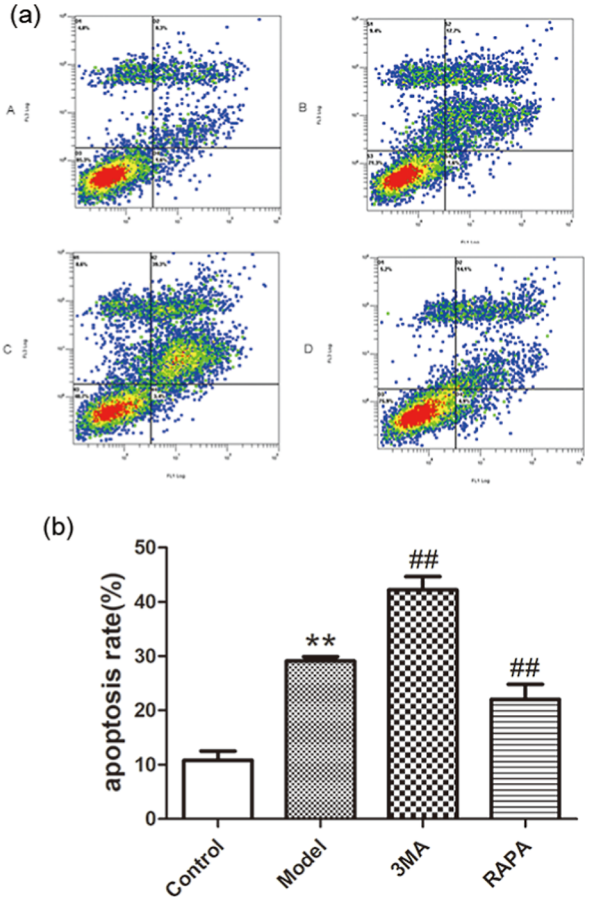

Figure 4. Effects of regulating autophagy on the apoptosis rate in HT22 cells subjected to OGD/R; (a) changes of the apoptotic rates in HT22 cells by OGD/R with different treatments examined by flow cytometry with Annexin V-FITC/PI staining; (b) graphical representation of quantified apoptosis by flow cytometry with Annexin V-FITC/PI staining. (A) Control; (B) model; (C) 3MA; (D) Rapa. (means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

compound C decreased *p*-AMPK/AMPK ($P < 0.01$). The expression of DDiT4 in the model group was increased significantly ($P < 0.01$). Compared with the model group, the expression of DDiT4 in the AICAR group was increased significantly, while the expression of DDiT4 in compound C group was decreased significantly ($P < 0.01$). *p*-mTOR/mTOR in the model group was decreased significantly and in AICAR group decreased further, while the *p*-mTOR/mTOR in compound C group was increased significantly ($P < 0.01$) (Figure 5). These data suggested that AMPK/DDiT4/mTOR axis was activated by OGD/R injury.

Level of autophagy after regulating the expression of AMPK/DDiT4/mTOR axis in HT22 cells subjected to OGD/R.

The level of autophagy was observed by a transmission electron microscope. The number of autophagy vesicles in the model group was increased significantly ($P < 0.01$) and was increased further by AMPK activator-AICAR ($P < 0.01$). There was no significant difference in the number of autophagy vesicles between the compound C group and the model group ($P < 0.01$) (Figure 6). The above result indicated that AMPK/DDiT4/mTOR axis regulates the activation of autophagy in HT22 cells after OGD/R.

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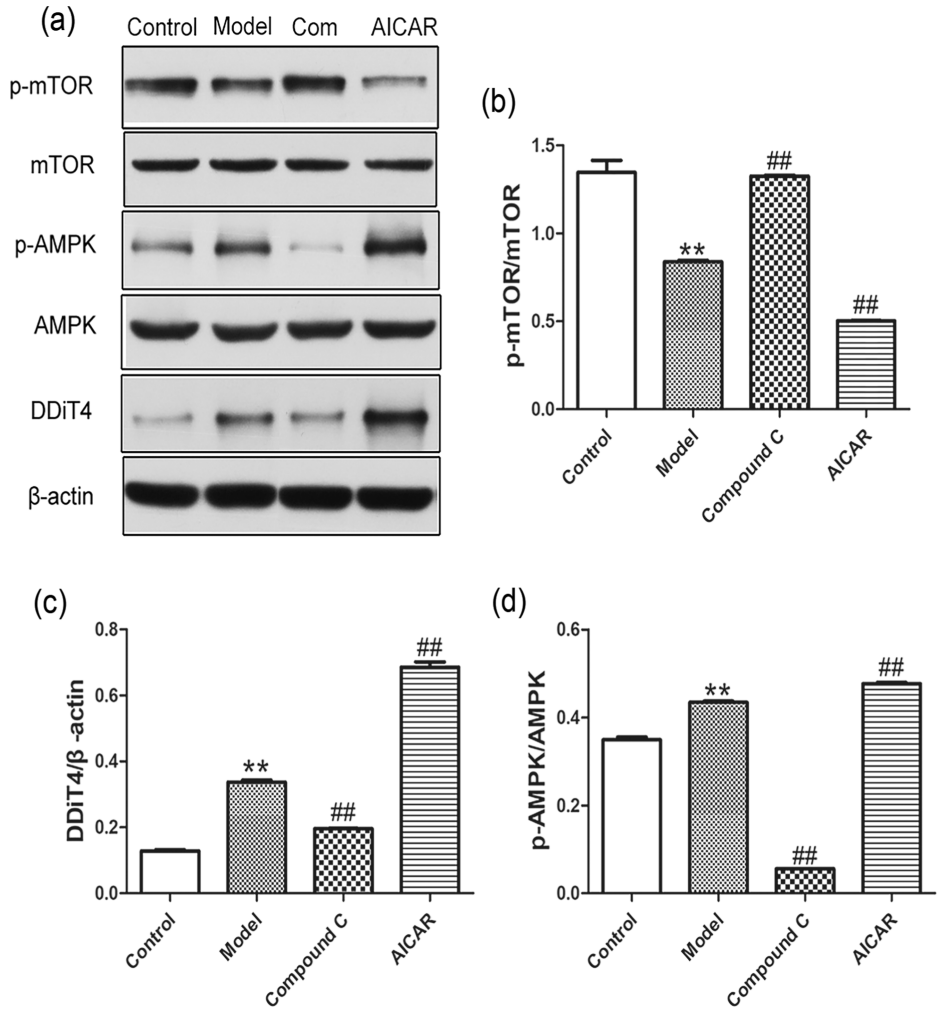

Figure 5. Level of AMPK/DDiT4/mTOR axis in HT22 cells after OGD/R injury (a) Western blot was used to analyze the expression of *p*-mTOR, mTOR, *p*-AMPK, AMPK, and DDiT4; (b) *p*-mTOR/mTOR was performed with Image J software; (c) banding of DDiT4 was quantified with Image J software; (d) *p*-AMPK/AMPK was performed with ImageJ software (means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

Effect of Regulating the Expression of AMPK/DDiT4/mTOR Axis on the Cell Viability after OGD/R Injury. The viability of HT22 cells was significantly reduced after OGD/R ($P < 0.01$), and the viability of HT22 cells was reduced further after treatment with AMPK inhibitor-compound C ($P < 0.01$). The viability of HT22 cells after OGD/R was significantly improved after treatment with AMPK activator-AICAR compared with model group ($P < 0.01$) (Figure 7). The result indicated that activating AMPK/DDiT4/mTOR axis is beneficial to promote the survival of cells after OGD/R.

Effects of Regulating the Expression of AMPK/DDiT4/mTOR Axis on the Levels of LDH in HT22 Cells Subjected to OGD/R. The level of LDH in OGD/R HT22 cells was significantly improved ($P < 0.01$), and the level of LDH in OGD/R HT22 cells was improved further after treatment with compound C ($P < 0.01$). However, the level of LDH decreased significantly in OGD/R HT22 cells after treatment with AICAR ($P < 0.01$) (Figure 8).

Effects of regulating the expression of AMPK/DDiT4/mTOR axis on the apoptosis in HT22 cells subjected to OGD/R.

TUNEL staining showed that marked apoptotic cells were detected in HT22 cells after OGD/R($P < 0.01$). AICAR repressed apoptosis in HT22 cells subjected to OGD/R ($P < 0.01$). While compound C significantly increased apoptosis significantly in OGD/R HT22 cells ($P < 0.01$) (Figure 9).

DISCUSSION

In this study, we elucidated that (1) autophagy can be activated by OGD/R and increasing the level of autophagy by rapamycin attenuates cell damage under OGD/R; (2) OGD/R activates AMPK/DDiT4/mTOR axis; activating AMPK/DDiT4/mTOR axis with AICAR, the activator of AMPK, increases the level of autophagy and is beneficial to alleviate cells injury caused by OGD/R. The above results indicated that AMPK/DDiT4/mTOR axis may be neuroprotective during OGD/R by mediating autophagy.

Autophagy, is shown to be vital for the maintenance of homeostasis in cells, is a "double-edged sword" in cell damage and recovery.[17] Accumulating pieces of evidence have shown that autophagy was induced in cerebral I/R injury in vivo and in vitro experiments.[18,19] In the present study, we found that OGD/R induced an increase in the number of autophagic vesicles in HT22 cells. Autophagy is a biological process involving a series of autophagy-related proteins.[20] We further explored the effect of OGD/R on autophagy-related proteins. Beclin1 is an important autophagy regulatory gene that recruits autophagy key protein to autophagic vesicle precursor, forming

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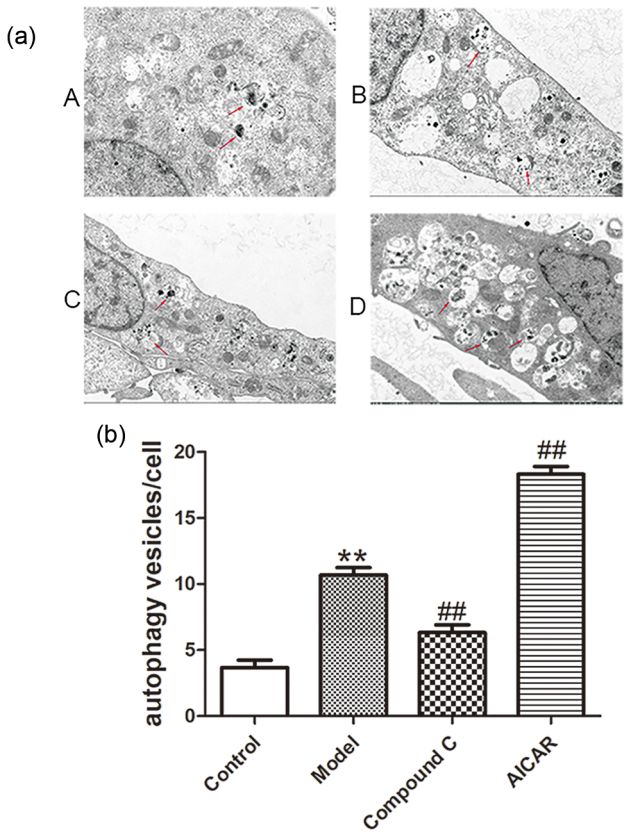

Figure 6. Level of autophagy after regulating the expression of AMPK/DDiT4/mTOR axis in HT22 cells subjected to OGD/R Autophagic vacuoles were observed by transmission electron microscopy; (A) control; (B) model; (C) compound C; (D) AICAR (means ± SD, $^{**}P < 0.01$ vs control and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model).

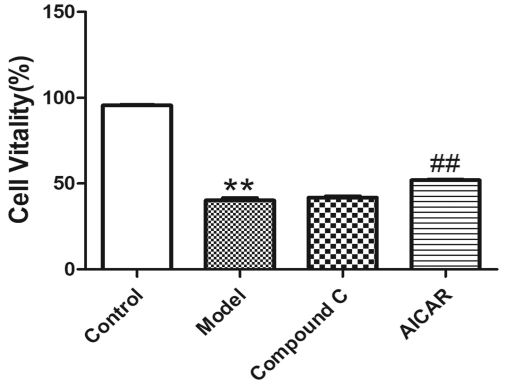

Figure 7. Effect of regulating the expression of AMPK/DDiT4/mTOR axis on the cell viability after OGD/R injury (data expressed as means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

a complex with Vps34 and Vps15; all of these provide an indispensable condition for the formation of autophagic vesicle.[21] LC3 includes LC3I and LC3II, and LC3II is a marker molecule of autophagic vesicles and serve as a read-out of an autophagic vesicles number.[22] P62 is the bridge connecting LC3 with the ubiquitinated substrate and will be degraded when autophagy occurs.[23] In this study, the expression of Beclin1, LC3I, LC3II, and P62 were tested. We found that autophagy was activated by OGD/R characterized by accumulation of Beclin1 and LC3II/LC3I

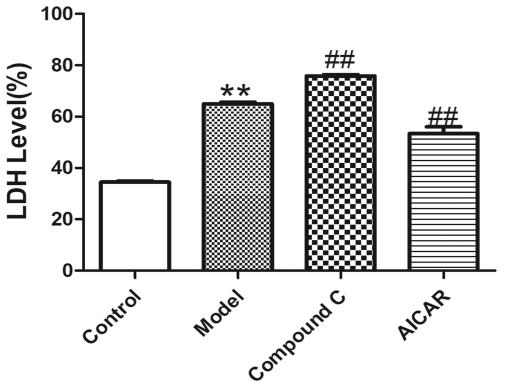

Figure 8. Effect of regulating the expression of AMPK/DDiT4/mTOR axis on the levels of LDH in HT22 cells subjected to OGD/R (data expressed as means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

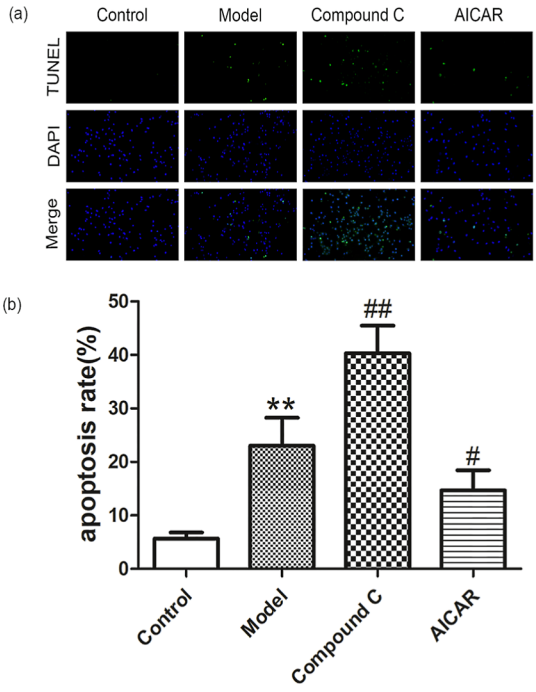

Figure 9. Effects of regulating the expression of AMPK/DDiT4/mTOR axis on the apoptosis in HT22 cells subjected to OGD/R (a) Changes of the apoptotic rates in HT22 cells by OGD/R with different treatments examined by TUNEL staining (x200), DAPI: bule; TUNEL: green; (b) graphical representation of apoptosis rate by TUNEL staining (means ± SD, $^{**}P < 0.01$ vs control and $^{\#\#}P < 0.01$ vs model).

and decreasing P62 expression, which were consistent with previous studies done by Liu.[24]

Plenty of research studies have indicated that autophagy plays an important role in cerebral I/R injury, and the role of autophagy in cerebral I/R injury is affected by various factors such as ischemia duration, ischemic phase, and cell types.[25,26] Zhou[27] and Chen[28] have confirmed that activating autophagy can effectively reduce neuronal damage caused by cerebral I/R injury through animal experiments. However, some researchers also put forward the opposite view.[29,30] We chose HT22 cells as the research object, and took oxygen and glucose deprivation/reoxygenation (OGD/R) 24 h as the time point to observe the role of autophagy in cerebral I/R injury. In our

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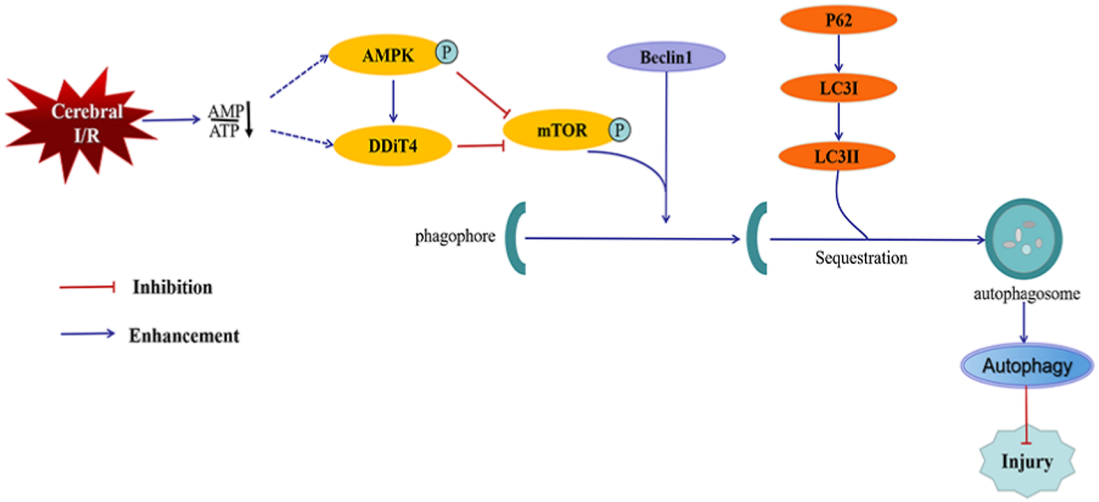

Figure 10. Neuroprotective mechanisms of autophagy against cerebral I/R injury.

research, we found that rapamycin (RAPA), the autophagy activator, increased cell viability and alleviated cell membrane damage and apoptosis by increasing the level of autophagy in OGD/R 24 h. In contrast, inhibition of autophagy by 3-methyladenine (3MA) led to apoptosis and the injury of HT22 cells aggravated. Therefore, the above results confirm that autophagy may have a protective effect on nerve cells in cerebral I/R 24 h, which are consistent with the study of Papadakis[31] and Wu.[32] The reason for the above results may be related to autophagy and provides a way for HT22 cells to eliminate damaged proteins, remaining organelles, and cytoplasm at an early stage of OGD/R.

AMPK, a serine/threonine protein kinase, is involved in the regulation of cellular energy metabolism and plays an important role in maintaining energy homeostasis.[33] When cells undergo ischemia and hypoxia, elevated AMP/ATP activates AMPK by increasing phosphorylation of Thr172 and autophagy dependent on the AMPK process that initiate to gain energy.[34] AMPK can phosphorylate TSC2 to inhibit mTOR signaling. mTOR, playing a key role in neurological disease, activates or inhibits autophagy by sensing changes in various signals inside and outside the cell, one of its complexes-mTORC1 directly inhibits autophagy.[35,36] DDiT4 is a negative regulator of mTOR that regulates cellular stress responses such as hypoxia and plays an irreplaceable role in the linkage between AMPK and mTOR signaling pathway.[37] It has been reported that the knockout of DDiT4 leads to a high defect in dephosphorylation of the key mTOR substrates S6K and 4E-BP1, resulting in the activation of AMPK failing to down-regulate mTOR activity.[38] As a downstream of AMPK, studies have shown that the expression of DDiT4 was increased and further enhanced by AMPK under hypoxic conditions. The increasing DDiT4 causes the TSC2/14-3-3 complex to depolymerize by binding to 14-3-3, thereby activating TSC1/2 and inhibiting mTORC1.[39] AMPK inhibits mTORC1 by activating TSC2, which is depolymerized by DDiT4, activates, and enhances autophagy.[40,41] Therefore, AMPK/DDiT4/mTOR axis plays an important role in regulating autophagy. However, the role of AMPK/DDiT4/mTOR axis in cerebral I/R injury has never been reported previously. In this study, we explored the role of AMPK/DDiT4/mTOR axis in cerebral I/R injury and the effect on autophagy during cerebral I/R injury of it. We found that *p*-AMPK/AMPK and protein level of DDiT4 were significantly increased, while *p*-mTOR/mTOR was significantly decreased in HT22 cells suffering OGD/R 24 h suggesting that AMPK/DDiT4/mTOR axis was activated by OGD/R. Then, we observed autophagy after changing the level of AMPK/DDiT4/mTOR axis by giving inhibitor and activator of AMPK in OGD/R HT22 cells; we found that the number of autophagic vesicles was increased in OGD/R HT22 cells after increasing the level of the axis by giving AMPK activator-AICAR. However, inhibition the level of axis by AMPK inhibitor-compound C exerted the opposite effect to the above. Finally, we explored the condition of OGD/R HT22 cells after changing the level of AMPK/DDiT4/mTOR axis. We found that viability was significantly increased, LDH leakage rate was reduced, and apoptosis was significantly repressed in ODG/R HT22 cells after increasing the level of AMPK/DDiT4/mTOR axis, suggesting that the neuroprotective effects of autophagy is associated with promoting of AMPK/DDiT4/mTOR axis activation (Figure 10).

In summary, our study provided experimental evidence that autophagy was induced by OGD/R and it has a protective effect on nerve cells in cerebral I/R 24 h, and its specific mechanism may be related to the activation of AMPK/DDiT4/mTOR axis. The above results could make a modification of autophagy a viable therapeutic strategy for protecting brain during ischemia and reperfusion. However, the role of autophagy at other time points in cerebral I/R injury is still not fully understood, and other mechanisms of autophagy in cerebral I/R injury need to be further explored.

MATERIALS AND METHODS

Cell Culture. HT22 cells (neuronal line of mouse hippocampus), presented by Professor Shunjiang Xu, were cultured in complete culture medium [DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Zhejiang Tianhang Biotechnology Co.,Ltd., Hangzhou, Zhejiang, China)]. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2; medium was changed every 2 days.[42] An in vitro model of I/R was built on HT22 cells.

Model of (OGD/R). Oxygen and OGD/R is an accepted in vitro model for simulating I/R injury.[43] HT22 cells were cultured in complete culture medium for 2 days and then the complete culture medium was discarded; cells were washed

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three times with PBS, added DMEM sugarless medium (Gibco, Grand Island, NY, USA), and maintained in the environment of 1% O_2, 5% CO_2, and 37 °C for 6 h. Then, DMEM sugarless medium was discarded and cells were washed three times with PBS, added complete culture medium, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h.

Experimental Design. Part I: the logarithmic growth phase of HT22 cells was randomly divided into four groups: control group, model group, 3MA group, and RAPA group. All the other groups of cells were treated with OGD/R except for the control group. Cells in the 3MA group were cultured in complete culture medium with 3-methyladenine (5 mmol/L) for 24 h, and cells in the RAPA group were cultured in complete culture medium with rapamycin (250 nmol/L) for 24 h[44] (Figure 11). 3-Methyladenine and rapamycin were provided by Sigma (St. Louis, MO, USA).

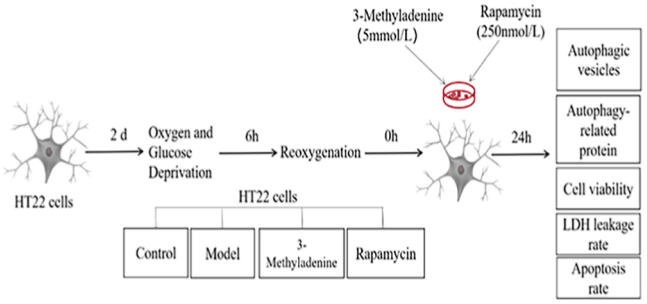

Figure 11. Grouping and detection methods in Part I.

Part II: the logarithmic growth phase of HT22 cells was randomly divided into four groups: control group, model group, compound C group and AICAR group. All the other groups of cells were treated with OGD/R except for the control group. Cells in the compound C group were cultured in complete culture medium with compound C (10 μmol/L)[45] for 24 h, and cells in the AICAR group were cultured in complete culture medium with AICAR (1 mmol/L)[46] for 24 h (Figure 12). Compound C and AICAR were provided by Sigma (St. Louis, MO, USA).

Cell Viability Analysis and Cytotoxicity Assay. WST-8 in CCK-8 is reduced to Formazan dye by dehydrogenase in cells under the action of 1-Methoxy PMS, and the more Formazan dyes produced, the more the number of living cells, so CCK-8 (Beijing Zoman Biotechnology Co.,Ltd., Beijing, China) can be used to measure viability of cells. HT22 cells were cultured in 96-well plates (5×10^4 cells/well) for 1 day and treated with OGD/R then. CCK-8 was added to HT22 cells (10 μL per well) for 1 h at 37 °C. The optical density was measured by Multi-Function Microplate Reader (Varioskan LUX, Thermo, USA) at an emission of 450 nm. Cell viability = (OD value of experimental group−OD value of blank group)/(OD value of control group−OD value of blank group) ×100%. LDH assay was used to detect cytotoxicity. LDH will be released from cells when cells are injured, so the content of LDH in the medium can infer the integrity of the cell membrane. The activity of LDH was measured at an emission of 450 nm. LDH leakage rate = LDH of cell supernatant/total LDH×100%.

Western Blot Analysis. Total cellular protein was extracted and detected by the BCA method; the proteins were denatured and separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane by the semi-dry transfer method, 5% off Lipid milk powder closed 2 h, anti-P62 (1:1000), anti-LC3 (1:1000), anti-Beclin1 (1:1000), anti-AMPK (1:1000), anti-*p*-AMPK (1:1000), anti-DDiT4 (1:1000), anti-mTOR (1:1000), and anti-*p*-mTOR (1:1000) were added at 4 °C overnight, secondary antibody (1: 3000) was incubated for 1 h at room temperature, and the optical density of the bands was measured by ImageJ software. Anti- P62 (GB11239-1) and anti-LC3 (GB11124) were obtained from Servicebio (Wuhan, Hubei, China). Anti-Beclin1 (PD017) was provided from MBL (Nagoya, Aichi, Japan). Anti-AMPK (2532S), anti-*p*-AMPK (2535S), anti-mTOR (2972S), and anti-*p*-mTOR (5536S) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-DDiT4 (ab106356) was purchased from Abcam (Cambridge, MA, USA).

Transmission Electron Microscopy. Cells were dissociated with 0.25% trypsin, centrifugated 5 min with 1500 r/min, and fixed for 4 h with 2.5% glutaraldehyde. The cells were washed by PBS three times and fixed by 1% citrate for 2 h. Specimens were embedded in embedding medium, and 50 nm thick sections were prepared after dehydration with acetone. After that, specimens were stained with uranyl acetate and lead acid. At last, morphological changes and numbers of autophagic vesicles in a unit area were observed by a transmission electron microscope (H-7650, Hitachi, Japan).

Flow Cytometry. Cells were dissociated by 0.25% trypsin, washed two times by PBS at 4 °C, centrifuged at 1500 r/min for 5 min, and resuspended by 100 μL 1×Binding Buffer (Contains 1×10^5 Cells). 5 μL FITC and 5 μL PI were added to cells after being resuspended, and the cells were incubated in

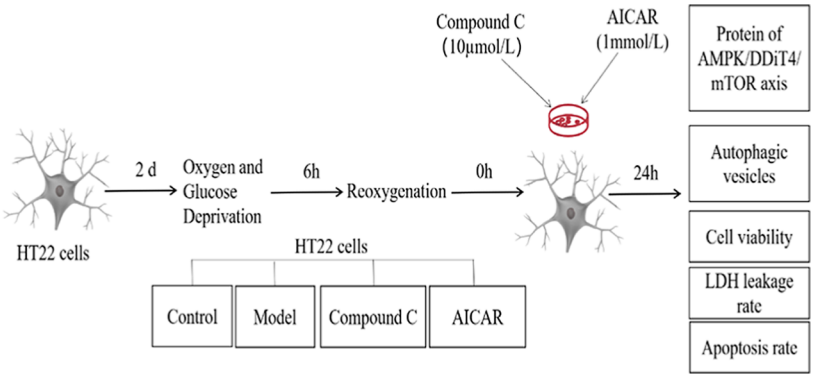

Figure 12. Grouping and detection methods in Part II.

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the dark for 15 min, and 400 μL 1×Binding Buffer was added. The rate of apoptosis was measured by a flow cytometer. Annexin V-FITC Apoptosis Detection Kit I was purchased from BD (BD Bio-Sciences Pharmingen, San Jose, CA).

TUNEL Staining. Apoptosis in the HT22 cells was assessed by the TUNEL assay using a TUNEL Apoptosis Assay kit (Roche, Basel, Switzerland). All nuclei were stained by DAPI and fluorescein-dUTP, and apoptotic nuclei were stained by TUNEL. The apoptotic index = the number of TUNEL-positive cells/the total number of cells.

Data Analysis. All data were analyzed by SPSS 23.0 software (SPSS Inc.) and presented as mean ± SD. One-way analysis of variance (ANOVA) was used to compare data between groups. $P < 0.05$ indicates statistical significance.

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Notes

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