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Attenuating oxygen-glucose deprivation-caused autophagosome accumulation may be involved in sevoflurane postconditioning-induced protection in human neuron-like cells

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Abstract

Application of the commonly used volatile anesthetic sevoflurane after brain ischemia (sevoflurane postconditioning) attenuates ischemic brain injury. It is not known whether autophagy plays a role in this sevoflurane postconditioning-induced neuroprotection. Human SH-SY5Y cells were induced to become neuron-like cells. These cells were subjected to 1 h oxygen-glucose deprivation (OGD) and then exposed to sevoflurane for 1 h. Chloroquine, an inhibitor of autolysosomes, rapamycin, an autophagy inducer, or 3-methyladenine (3-MA), an autophagy inhibitor, were incubated with cells during OGD and sevoflurane exposure. OGD and the subsequent simulated reperfusion increased lactate dehydrogenase (LDH) release from the cells. This increase was dose-dependent inhibited by sevoflurane postconditioning. OGD increased the ratio of microtubule-associated protein 1 light chain 3 (LC3) II to LC3I and the expression of beclin-1 and p62. These increases were attenuated by sevoflurane. Sevoflurane alone did not have any effects on the expression of p62, beclin-1 and the ratio of LC3II to LC3I. Sevoflurane also enhanced the co-location of autophagosomes and lysosomes. Chloroquine increased the ratio of LC3II to LC3I, p62 and LDH release in cells subjected to OGD. Sevoflurane postconditioning attenuated OGD-induced inactivation of Akt and mechanistic target of rapamycin (mTOR). Inducing autophagosome generation by rapamycin attenuated sevoflurane postconditioning-

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reduced LDH release. Inhibition of autophagosome generation by 3-MA decreased OGD-induced LDH release. These results suggest that OGD increase autophagosome accumulation via increased formation of autophagosomes and reduced autophagosome clearance and that attenuation of OGD-induced autophagosome accumulation may contribute to sevoflurane postconditioning-induced cell protection.

Keywords

autophagy; autolysosomes; neuroprotection; sevoflurane

1. Introduction

Stroke remains a major global health problem. The Global Burden of Disease (GBD) study ranked stroke as the second most common cause of death (Lozano, et al., 2012) and the third most common cause of disability-adjusted life-years (DALYs) (Murray, et al., 2012) worldwide in 2010. Despite stable incidence rates and declining mortality rates over the past 20 years, the absolute number of people who have a stroke every year, live with the consequences of stroke, and die from their stroke is increasing (Feigin, et al., 2014). The high mortality and disability rates of stroke urge researchers to seek effective treatment. While the majority of clinical trials on exogenous neuroprotective agents for stroke have been disappointing (Ehrenreich, et al., 2009; Ikonomidou, et al., 2002), evoking the endogenous ability for neuroprotection, such as via ischemic conditioning, is a focus of research (Pan, et al., 2016).

Ischemic conditioning refers to a number of related neuroprotective strategies that render neural tissues tolerant to ischemia by conditioning them with brief cycles of ischemia and reperfusion (Murry, et al., 1986), or with some other treatments, such as hypoxia (Zhao, et al., 2005) and volatile anesthetic (Adamczyk, et al., 2010). Although the field of conditioning was started with ischemic preconditioning (brief cycles of ischemia and reperfusion before a long duration of ischemia) (Murry, et al., 1986), its clinical application is limited because the intervention requires accurate prediction of the occurrence of the severe ischemic events. Compared with preconditioning, postconditioning that involves using the conditioning stimulus after the damaging insult has already occurred can also provide neuroprotection (Zhao, et al., 2003). Among the methods that can induce this effect, anesthetic postconditioning has attracted special attention because it is a non-invasive maneuver (Lee, et al., 2008). As a newer volatile anesthetic that is used worldwide in patient care, sevoflurane has been shown to induce a postconditioning effect in the brain (Ren, et al., 2014).

Autophagy is an important physiological process that maintains cellular homeostasis through lysosome-mediated protein degradation and organelle turnover (Mizushima, 2007). Recent studies have shown that autophagy is involved in the cardioprotective effects of ischemic preconditioning and cell protection by the general anesthetic isoflurane (Gidlof, et al., 2016). Anesthetics have effects on autophagy (Ye, et al., 2017). Thus, we hypothesize that the regulation of autophagy is involved in the neuroprotection conferred by sevoflurane

postconditioning. To test this hypothesis, we induced human SH-SY5Y cells into neuron-like cells. Oxygen-glucose deprivation (OGD) was used to simulate ischemia *in vitro*.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cells, a human neuroblastoma cell line, were obtained from the American Type Culture Collection (Manassas, VA) and cultured as we described before (Lin, et al., 2011). Briefly, cells were cultured in a T75 flask containing 13 ml of Eagle's Minimum Essential Medium (EMEM)/Ham's F-12 nutrient mixture (1:1) with 10% fetal bovine serum. The cells were kept at 37°C in a humidified incubator gassed with 95% air and 5% CO_2. The medium was changed twice per week. When the cells were 70% - 80% confluent, they were exposed to 0.25% trypsin-EDTA solution and sub-cultured in a new flask.

The SH-SY5Y cells were plated at a density of 1×10^5 cells/cm^2 in 6-well plates for lactate dehydrogenase (LDH) release assay or Western blotting. One day after plating, cells were incubated in neurobasal medium, supplemented with B-27 supplement (GIBCO, Carlsbad, CA) and L-glutamine (500 μM; Nacalai Tesque Inc., San Diego, CA). Retinoic acid (RA, Sigma, St Louis, MO) at 10 μM was added to the medium for 3 days to induce SH-SY5Y cells to differentiate into a homogenous population of cells with neuronal morphology (Kume, et al., 2008). These cells were used in the experiments.

2.2. Oxygen-glucose deprivation

Cells in control group were washed with phosphate buffered saline (PBS) and incubated in neurobasal medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The exposure of cells to OGD was performed as we described before (Wu, et al., 2010). Briefly, neurobasal-A medium that did not contain L-glucose (GIBCO) was bubbled with 100% N_2 for 30 min. Cells were washed with PBS once and 2 ml/well of the neurobasal-A medium was added to the cells. These plates were immediately placed in an air-tight chamber (Billups-Rothenberg, Inc., Del Mar, CA) gassed with 100% N_2 for 10 min. The oxygen content in the outlet of the chamber was monitored with a DatexTM infrared analyzer (Capnomac, Helsinki, Finland) and reached 2% at 3 – 5 min after the onset of gassing. After closure of the inlet and outlet of the chamber, the chamber was kept at 37°C for 1 h. After confirming that the oxygen content in the chamber was still lower than 2% at the end of the OGD period, the chamber was opened and glucose, B-27 supplement and L-glutamine (500 μM) was added to make the final glucose concentration in the buffer at 4.5 g/l. The plates were kept for 6 h (Western blotting) or 20 h (Western blotting and LDH release assay) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.3. Anesthetic exposure

Cells were placed in an airtight chamber immediately after OGD. The chamber was gassed with 1%, 2%, 3%, 4% or 5% sevoflurane in the carrying gases (95% air and 5% CO_2) for 15 min. Volatile anesthetic concentrations in the gases from the outlet of the chamber were monitored with a DatexTM infrared analyzer and reached the target concentrations at ~3 min after the onset of gassing. The chamber was sealed and the incubation was for 1 h at 37°C.

At the end of incubation, anesthetic concentrations in the gases from the chamber were confirmed to be at the target concentrations by the infrared analyzer. The plates then were kept for 5 h or 19 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.4. Treatment with other reagents

The stock solution of chloroquine diphosphate (CQ) (Sigma, C6628) was prepared in PBS and stored at −20°C until it was used. To block autophagosome clearance, cells in 6-well plates were treated with 50 μM CQ (Pivtoraiko, et al., 2010) at the beginning of OGD or at the corresponding time point for the control group. After the OGD, cells were incubated with CQ at 37°C until they were used for Western blot or LDH assay.

To study the role of autophagy activation, cells were pretreated with rapamycin for 24 h. The final concentration of rapamycin (Sigma, R0395) was 0.2 μM (final concentration of DMSO that was used to dissolve rapamycin was 0.1% v/v) (Xiong, et al., 2011). DMSO was added to the culture medium in other groups. Rapamycin was present until the cells were harvested for assay.

To investigate the effect of autophagy inhibition, 3-methyladenine (3-MA, Sigma, M9281) was dissolved in PBS at 55°C, stored at −20°C and completely dissolved by warming to 55°C just prior to use. The final concentration of 3-MA was 5 mM (Deng, et al., 2013). 3-MA was added 1 h prior to OGD and present until cells were harvested for assy.

2.5. LDH activity assay

LDH activity was determined using an LDH cytotoxicity detection kit as we did before (Kim, et al., 2009a; Kim, et al., 2009b). Briefly, the incubation solution harvested at the end of experiments was centrifuged at 16,060 g for 10 min. One hundred micro-litters of the supernatant were transferred to 96-well plates and incubated with the same volume of reaction mixture from the kit. The samples then were read in a spectrophotometry (Bio-Rad Laboratories, Hercules, CA) with the absorbance wavelength at 492 nm and the reference wavelength at 655 nm. Background absorbance from the cell-free buffer solution was subtracted from all absorbance measurements. After removal of the incubation solution from 6-well plates, 1% triton X-100 lysing solution was applied to each well to dissolve the remaining cells. The percentage of LDH released to incubation buffer in total LDH was calculated as follows: LDH in the buffer × 100 / (LDH in the buffer + intracellular LDH released by triton X-100).

2.6. Western blotting

Human neuron-like cells after various treatments were homogenized in RIPA buffer (Thermo Scientific, 89901) containing protease inhibitor cocktail (Sigma, P2714). The phosphatase inhibitor (Roche, 04906845001) was added when preparing samples for detecting phospho-Akt and phospho-mTOR. Homogenates were centrifuged at 4°C for 20 min at 16,060 g. Protein content in the supernatant was determined using a Bio-Rad Protein Assay Kit. Twenty micrograms of proteins per lane were loaded and electrophoresed in a 12% polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane. The membrane was blocked with Protein-Free T20 Blocking Buffer (Thermo Scientific, 37573)

and then incubated with the following primary antibodies: rabbit anti-LC3B (1:500; Abcam, ab48394) antibody, rabbit anti-beclin-1 (1:1000; Cell Signaling, D40C5) antibody, mouse anti-p62 (1:1000; Abcam, ab56416) antibody, goat anti-cathepsin D (1:200; Santa Cruz, sc-6486) antibody, mouse anti-actin (1:5000; Abcam, ab6276) antibody, rabbit anti-phospho-mTOR (Ser2448) (1:1000, Cell Signaling, 2971) antibody, mouse anti-mTOR (1:1000, Cell Signaling, 4517) antibody, mouse anti-phospho-Akt (Ser473) (1:1000, Cell Signaling, 12694) antibody, or rabbit anti-Akt (1:1000, Cell Signaling, 4685) antibody. Appropriate secondary antibodies were used and protein bands were visualized and quantified using G:Box equipped with gene tools analysis software (Syngene, Frederick, MD, USA). The densities of protein bands were normalized to those of actin to control for errors in protein sample loading and transferring during Western blotting. The results from cells under various experimental conditions then were normalized by those of the corresponding control cells.

2.7. Immunofluorescent staining

Human neuron-like cells plated on Lab-Tek Chamber slides (Thermo Fisher Scientific, 177445) after various treatments at 20 h after the OGD were used for staining. They were washed once with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After being washed with PBS for 3 times, cells were incubated with PBS containing 0.1% Triton X-100 for 30 min and washed with PBS for 3 times again. The cells were incubated with blocking solution in PBS containing 5% bovine serum albumin (BSA, MP Biomedicals, 820451) for 1 h at room temperature. Next, they were incubated with appropriate primary antibodies [rabbit anti-LC3B antibody, 1:250, Abcam, ab48394; mouse anti-lysosome-associated membrane glycoprotein 1 precursor −1 (LAMP-1) antibody, 1:200, Abcam, ab25630] diluted in PBS with 5% BSA for 24 h at 4°C. Slides were then rinsed with PBS for 3 times. Cells were subsequently incubated with Alexa Fluor 488 (1:200 dilution, Invitrogen, A21206) or 594 (1:200 dilution, Invitrogen, A21203) secondary antibody in PBS with 5% BSA for 1 h at room temperature in the dark. After being rinsed with PBS for 3 times again, nuclei were stained with DAPI solution (Thermo Fisher Scientific, 62249) for 3 min at room temperature. Cells were washed 3 times with PBS, and covered with coverslips in Aqua-Mount mounting medium (Richard-Allan Scientific, 13800) and left at 4°C overnight.

2.8. Confocal microscopy and co-localization analysis

The slides were examined under an Axio Observer LSM 710 confocal system (Carl Zeiss Microscopy GmbH, Jena, Germany) and analyzed with ZEN Black software (Carl Zeiss Microscopy GmbH). Co-localization analysis of images acquired by confocal laser scanning microscopy was performed using the software FIJI (ImageA 1.45j; Max Planck Society). The plugin “Coloc 2” allows the quantitative determination of co-localizing fluorescence intensities acquired in different channels using methods described previously (Costes, et al., 2004; Manders, et al., 1993). The obtained Mander’s coefficients were used as the fraction of co-localization of both channels, i.e. tM1 represented the fraction of lysosomes that also contain green fluorescent protein LC3 and tM2 represented the fraction of pixels of green signal from LC3 that also had red signal from lysosomes. In addition to the Mander’s coefficients, Pearson correlation coefficients were obtained as another measure for co-

localization. One hundred fifty cells in random fields from 3 independent experiments were analyzed.

2.9. Statistical analysis

Results are presented as mean ± S.E.M. ($n \geq 5$). Data were analyzed by one-way analysis of variance followed by Tukey test if the data were normally distributed, by one-way analysis of variance on ranks followed by Tukey test if the data were not normally distributed. Differences were considered to be significant at $P < 0.05$ based on two-tailed hypothesis testing. All statistical analyses were performed with SigmaStat 3.5 (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

3.1. Sevoflurane protected neuron-like cells and CQ enhanced cell injury after OGD-reperfusion

As shown in Fig. 1, the human neuron-like cells differentiated from SH-SY5Y cells responded to OGD and simulated reperfusion with an increase of LDH release into culture medium, suggesting cell injury. This injury was dose-dependently decreased by sevoflurane applied for 1 h immediately after OGD. However, the effects of sevoflurane appeared to be bell-shaped curve with the best protective effect at 2% sevoflurane, a clinically relevant concentration. Based on these results, 2% sevoflurane was used for all subsequent experiments. To determine whether autophagosome-lysosome fusion and autophagosomes play a role in cell injury, we used CQ, a well-known inhibitor of autophagic proteolysis (Kimura, et al., 2013). CQ treatment significantly aggravated the injury of neuron-like cells after OGD-reperfusion injury no matter whether this was in the presence or absence of sevoflurane postconditioning (Fig. 1B). These results suggest the importance of normal autophagy in cell survival.

3.2. Sevoflurane promoted autophagosome clearance in cells after OGD

To determine the role of autophagy in the protective effects of sevoflurane, an analysis of the expression of the autophagic markers LC3, beclin-1, p62 and cathepsin D was conducted at 6 h and 20 h after OGD. The ratio of LC3II/LC3I and the expression of beclin-1 and p62 were increased at both time points. This increase was reduced by 2% sevoflurane postconditioning. However, either OGD-reperfusion or 2% sevoflurane postconditioning did not affect the level of cathepsin D (Fig. 2). Because LC3 conversion is a marker for autophagosome formation (Glick, et al., 2010; Mizushima, 2007) and p62 is selectively incorporated into autophagosomes through direct binding to LC3 and then degraded by autophagy (Glick, et al., 2010; Mizushima, 2007; Rusten, et al., 2010), these results suggest that OGD impairs the clearance of autophagosomes and sevoflurane attenuates this impairment. However, the OGD-reperfusion-induced impairment of autophagosome clearance is not likely due to the dysregulation of lysosomal activity because the expression of cathepsin D, an important proteinase contained in lysosomes (Barrett, 1970), was not changed by OGD or sevoflurane compared with control group. We used CQ to clarify the role of sevoflurane-induced autophagosome clearance enhancement in the protection of sevoflurane. Like NH_4Cl, CQ neutralizes the lysosomal pH and causes the accumulation of

sequestrated materials in either autophagosomes or autolysosomes (Dunmore, et al., 2013; Geng, et al., 2010). Moreover, CQ has been suggested to block autophagosomes fusion with lysosomes (Klionsky, et al., 2016). CQ increased LC3II and p62 (Fig. 2), suggest that the maturation of autophagosomes has been blocked in the presence of CQ. CQ did not change the expression of beclin-1, suggesting that CQ does not affect the induction of autophagosome formation. In addition, CQ reduced the expression of cathepsin D (Fig. 2), consistent with its effects on lysosomes. Interestingly, sevoflurane did not affect the expression of p62, beclin-1 and the ratio of LC3II to LC3I under control condition (Fig. 3), suggesting that sevoflurane does not alter the formation and clearance of autophagosomes under control condition.

3.3. Sevoflurane improved autophagosome-lysosome fusion

To better understand how sevoflurane improved autophagosome clearance, we examined autolysosome formation. Autophagosomes were visualized via LC3 puncta (green fluorescence), and lysosomes were visualized using LAMP-1 staining (red fluorescence). LAMP-1 is a lysosomal protein (Carlsson, et al., 1989). Quantification data showed that OGD-reperfusion significantly decreased the numbers of autolysosomes (positive staining for both LC3 and LAMP-1) compared with control group. Sevoflurane postconditioning increased the co-localization rate of LC3 with LAMP-1 (Fig. 4). These results indicate that 2% sevoflurane postconditioning improves autophagosome-lysosome fusion.

3.4. Sevoflurane attenuated OGD-induced inactivation of Akt-mTOR pathway to inhibit autophagosome accumulation to protect human neuron-like cells against OGD

The reduction of OGD-reperfusion-induced accumulation of autophagosomes by sevoflurane could be caused by either a decrease in autophagosome generation or increase of autolysosome clearance. Thus, we also examined whether sevoflurane maintained the activation of the Akt-mTOR signaling because activation of this signaling could reduce autophagosome production. Fig. 5 showed that the levels of p-Akt and p-mTOR in the OGD-reperfusion group were lower than that of control group. Sevoflurane postconditioning significantly increased the expression of p-Akt and p-mTOR. In addition, rapamycin, an mTOR inhibitor, attenuated sevoflurane postconditioning-induced decrease of LC3 conversion (Fig. 5). These results suggest that sevoflurane maintains the activation of Akt-mTOR signaling to reduce the generation of autophagosomes under OGD condition.

Consistent with its known effects, 3-MA reduced autophagosome production as reflected by a decrease in the LC3 conversion. Interestingly, 3-MA decreased OGD-reperfusion-induced cell injury and rapamycin attenuated the protective effects of sevoflurane (Fig. 5). Also, rapamycin further increased the ratio of LC3II to LC3I and LDH release in cells with OGD (Fig. 6). These results suggest that the reduced autophagosomes generation may be involved in the protective effects of sevoflurane postconditioning.

4. DISCUSSION

Postconditioning with sevoflurane has been shown to induce protection in rodent brains (Ren, et al., 2014). Our current study suggests that sevoflurane postconditioning also protect

human neuron-like cells. Here, we showed that a concentration as low as 1% sevoflurane for 1 h applied at the onset of simulated reperfusion was sufficient to reduce cell injury. This protection appeared to peak at 2% sevoflurane. Higher concentrations had a reduced protective effect. These results suggest that sevoflurane at clinically relevant concentrations induces postconditioning effects in human neuron-like cells. The reasons for the decreased protective effect with sevoflurane concentrations higher than 2% are not yet known. One possibility for this phenomenon is that sevoflurane at high concentrations may injure cells, which can partially offset sevoflurane-induced protective effects. For example, exposure to 3% sevoflurane for 2 h for 3 consecutive days leads to significant cognitive impairment with increased neuronal apoptosis and overexpression of neuroinflammatory markers in neonatal mice (Xia, et al., 2017).

There was a significant increase in the levels of autophagic markers (LC3 conversion) in the human neuron-like cells after OGD. One of the possible reasons for OGD-induced increase in autophagic markers is the increase of autophagosome generation. The activation of the Akt-mTOR pathway is known to inhibit autophagosome generation in response to various stresses in different cells (Ryou, et al., 2015; Saiki, et al., 2011). Our results showed that OGD led to a significant decrease in the levels of phosphorylated Akt and mTOR, which suggests that OGD increases autophagosome generation. Consistent with this suggestion, OGD also increases beclin-1, a protein that is involved in inducing autophagosome formation (Glick, et al., 2010; Mizushima, 2007). Sevoflurane postconditioning increases the levels of phosphorylated Akt and mTOR in cells after OGD and rapamycin, an mTOR inhibitor (Lamming, et al., 2012), attenuated sevoflurane effects on LC3 conversion. Rapamycin further increased cell injury and autophagosome generation under OGD condition. Also, sevoflurane decreased beclin-1 under OGD condition but did not affect the expression of p62, beclin-1 and the ratio of LC3II to LC3I under control condition. These results suggest that sevoflurane postconditioning decreased OGD-induced increase of autophagosome generation possibly via attenuating inactivation of Akt-mTOR pathway.

The increase of LC3II level by OGD may also be caused by blockage in autophagosomal maturation and degradation (Mizushima, et al., 2010). Therefore, we examined the changes of autophagic flux by tracking the p62 protein level. p62 is selectively incorporated into the autophagosome through direct binding to LC3 and is degraded by autophagy (Saiki, et al., 2011). Here, we showed that OGD increased p62 protein level and that sevoflurane postconditioning prevented this effect. These results indicate that the increased autophagic marker (LC3II) in OGD-treated cells is not only due to enhanced autophagosome generation but also by suppression of the autophagosome maturation and degradation. Sevoflurane postconditioning promoted the degradation of autophagosomes accumulated in the cells after OGD.

To complete the autophagic process, autophagosomes need to be fused with lysosomes to form autophagolysosomes and its components are degraded by lysosomal hydrolases. The metabolites are transported to the cytosol for reuse (Zhou, et al., 2012). In this process, the autophagosome-lysosome fusion, lysosomal proteases and lysosomal pH are critical factors to complete the degradation (Shen, et al., 2014). We examined fluorescence co-localizations of punctate autophagosomal labeling (LC3) and the lysosomal labeling (LAMP1). Our study

showed that OGD reduced the co-localization of LC3 with LAMP1 and that sevoflurane improved this co-localization, suggesting that OGD impairs the fusion of autophagosome with lysosome and that sevoflurane postconditioning attenuates this impairment.

Our study does not appear to show a significant effect on the enzymes of lysosomes by current OGD and simulated reperfusion condition because the level of cathepsin D was not changed by OGD. Sevoflurane postconditioning also did not affect cathepsin D expression. In addition, CQ that can increase lysosomal pH to inhibit lysosomal enzymes (Dunmore, et al., 2013; Geng, et al., 2010) decreased cathepsin D and also further increased the ratio of LC3II to LC3I and p62 no matter whether sevoflurane postconditioning was presented. These results suggest that the current conditions of OGD and sevoflurane postconditioning may not have significant effects on lysosomal pH and enzymes to change the autophagosome clearance.

Previous studies show that impaired autophagosome clearance plays a critical role in many diseases. Impaired autophagosome clearance contributes to neuronal death in a piglet model of neonatal hypoxic-ischemic encephalopathy (Cui, et al., 2017). Autophagosome clearance was markedly impaired in the diabetic heart subjected to myocardial ischemia-reperfusion and increasing autophagosome clearance reduced infarct size and improved cardiac function (Wang, et al., 2017b). Also, autophagy dysregulation or autophagosome accumulation occurs in ischemic spinal cord of rats and traumatic brain injury in mice and appears to contribute to the spinal cord and brain injury in these animals (Liu, et al., 2015; Sarkar, et al., 2014). As discussed above, our results suggest that sevoflurane postconditioning facilitates autophagosome-lysosome fusion and reduces the formation of autophagosome and, therefore, attenuates OGD-induced autophagosome accumulation. Thus, these effects shall contribute to sevoflurane postconditioning-induced cell protection. To support this possibility, rapamycin increased autophagosome formation and attenuated sevoflurane postconditioning-induced cell protection. Inhibition of autophagosome formation by 3-MA protected the human neuron-like cells against OGD.

Our results may not support that sevoflurane has a direct effect on autophagy under control condition because sevoflurane alone did not affect the expression of p62, beclin-1 and the ratio of LC3II to LC3I. However, our data indicate that sevoflurane postconditioning may reduce OGD-induced autophagosome accumulation to attenuate cell injury because OGD induced autophagosome accumulation and cell injury, sevoflurane postconditioning attenuated this accumulation and provided neuroprotection, and increasing autophagosome formation by rapamycin or deceasing autophagosome clearance by chloroquine attenuated sevoflurane postconditioning-induced reduction of autophagosome accumulation and neuroprotection. Mechanistically, sevoflurane postconditioning may decrease OGD-induced autophagosome accumulation through signaling events that regulate autophagy because sevoflurane attenuated OGD-induced inactivation of mTOR and inhibition of mTOR by rapamycin reduced sevoflurane-induced autophagy and neuroprotection. Nevertheless, attenuating autophagosome accumulation may be a downstream focused event for sevoflurane postconditioning-induced cell protection.

Our results suggest the effects on autophagy as a novel mechanism for sevoflurane postconditioning-induced cell protection. Increased autophagy has been suggested as a mechanism for isoflurane-induced protection in the brain and liver (Rao, et al., 2017; Sheng, et al., 2014). Increased autophagy has also been indicated as a mechanism for electroacupunctureor hyperbaric oxygen-induced neuroprotection (Fang, et al., 2017; Yan, et al., 2011). However, decreased autophagy has been proposed to mediate the neuroprotective effects of electroacupuncture, ketamine and dexmedetomidine (Luo, et al., 2017; Wang, et al., 2017a; Wu, et al., 2015). The reasons for these contradictory conclusions are not known. However, it is known that proper autophagy may be beneficial to save energy and clean damaged organelles and excessive autophagy contributes to cell death caused by ischemia-reperfusion injury (Koike, et al., 2008; Wang, et al., 2016). Thus, achieving appropriate levels of autophagy is needed for cell protection (Ren, et al., 2017). Our results with sevoflurane postconditioning suggest the importance of this delicate balance.

In summary, the present study has shown that OGD and simulated reperfusion increased autophagosome generation and reduced autophagosome clearance to enhance the accumulation of autophagosomes. Sevoflurane postconditioning improves autophagosome clearance and inhibits autophagosome generation via attenuating the inactivation of Akt-mTOR pathway under OGD condition. These sevoflurane effects on autophagy may be responsible for its neuroprotection.

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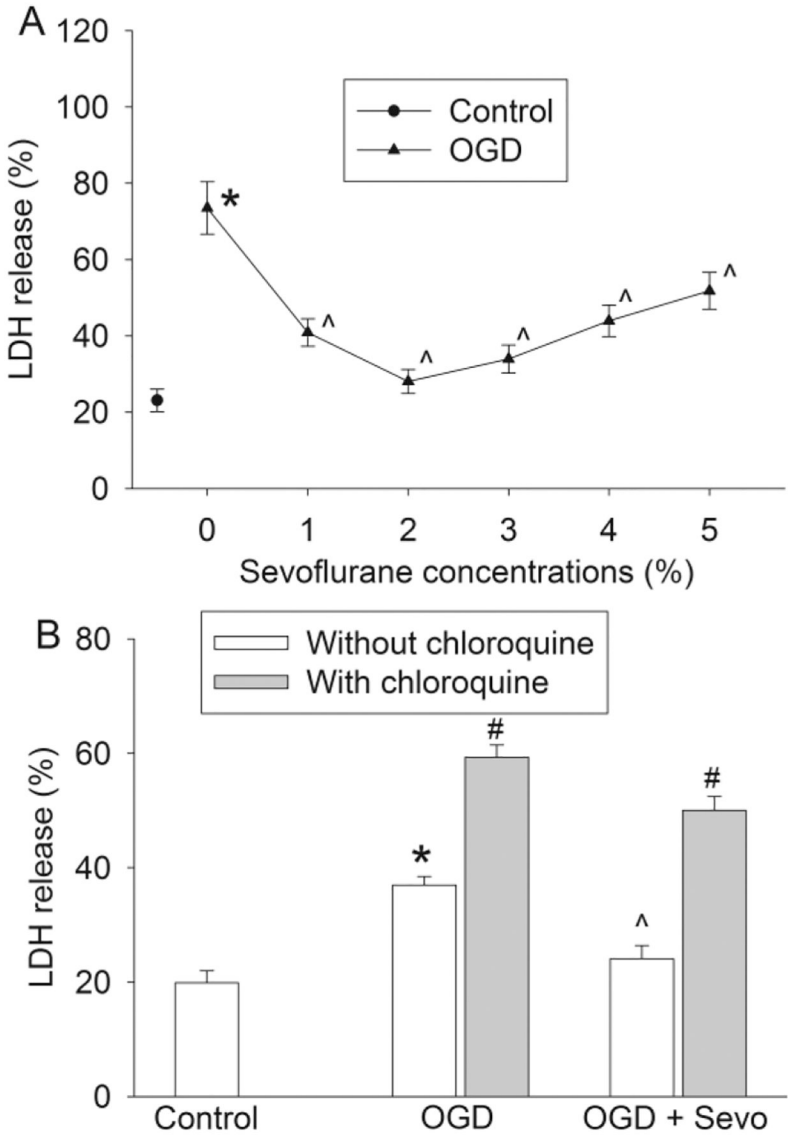

Fig. 1. Sevoflurane attenuated oxygen-glucose deprivation (OGD)-induced cell injury and chloroquine enhanced the injury.

Neuron-like cells were subjected to 1 h OGD followed with a 20 h simulated reperfusion. A: Cells were exposed to various concentrations of sevoflurane for 1 h immediately after the onset of simulated reperfusion. Results are means ± S.E.M. (n = 18). B: Chloroquine was added just before the OGD and was present till the cells were harvested for LDH release assay. Sevoflurane at 2% was used in this experiment. Results are means ± S.E.M. (n = 9). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD group. # $P < 0.05$ compared with the corresponding condition without chloroquine. OGD: oxygen-glucose deprivation; Sevo: 2% sevoflurane

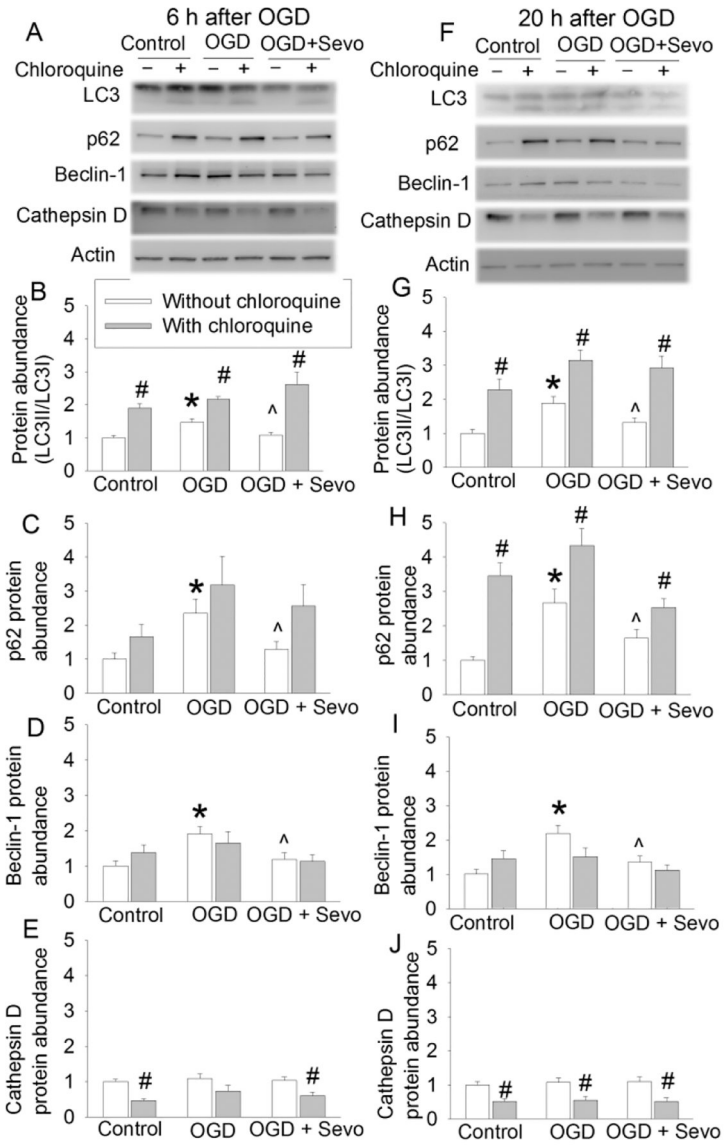

Fig. 2. Effects of 2% sevoflurane postconditioning on OGD-induced autophagic markers in human neuron-like cells.

Cells were subjected to 1 h OGD followed with 6 h or 20 h simulated reperfusion. Cells were exposed to 2% sevoflurane for 1 h immediately after the onset of simulated reperfusion. Chloroquine was added before the OGD and was present till the cells were harvested for assay. A: Representative images of Western blotting of samples harvested at 6 h after OGD. B: Quantitative results of the ratio of LC3II to LC3I at 6 h after OGD. C: Quantitative results of p62 at 6 h after OGD. D: Quantitative results of beclin-1 at 6 h after OGD. E: Quantitative results of cathepsin D at 6 h after OGD. F: Representative images of Western blotting of samples harvested at 20 h after OGD. G: Quantitative results of the ratio of LC3II to LC3I at 20 h after OGD. H: Quantitative results of p62 at 20 h after OGD. I: Quantitative results of beclin-1 at 20 h after OGD. J: Quantitative results of cathepsin D at 20 h after OGD. Results are means ± S.E.M. (n = 9). * $P < 0.05$ compared to control. ^ P <

0.05 compared to OGD group. # $P < 0.05$ compared with the corresponding condition without chloroquine. OGD: oxygen-glucose deprivation; Sevo: 2% sevoflurane.

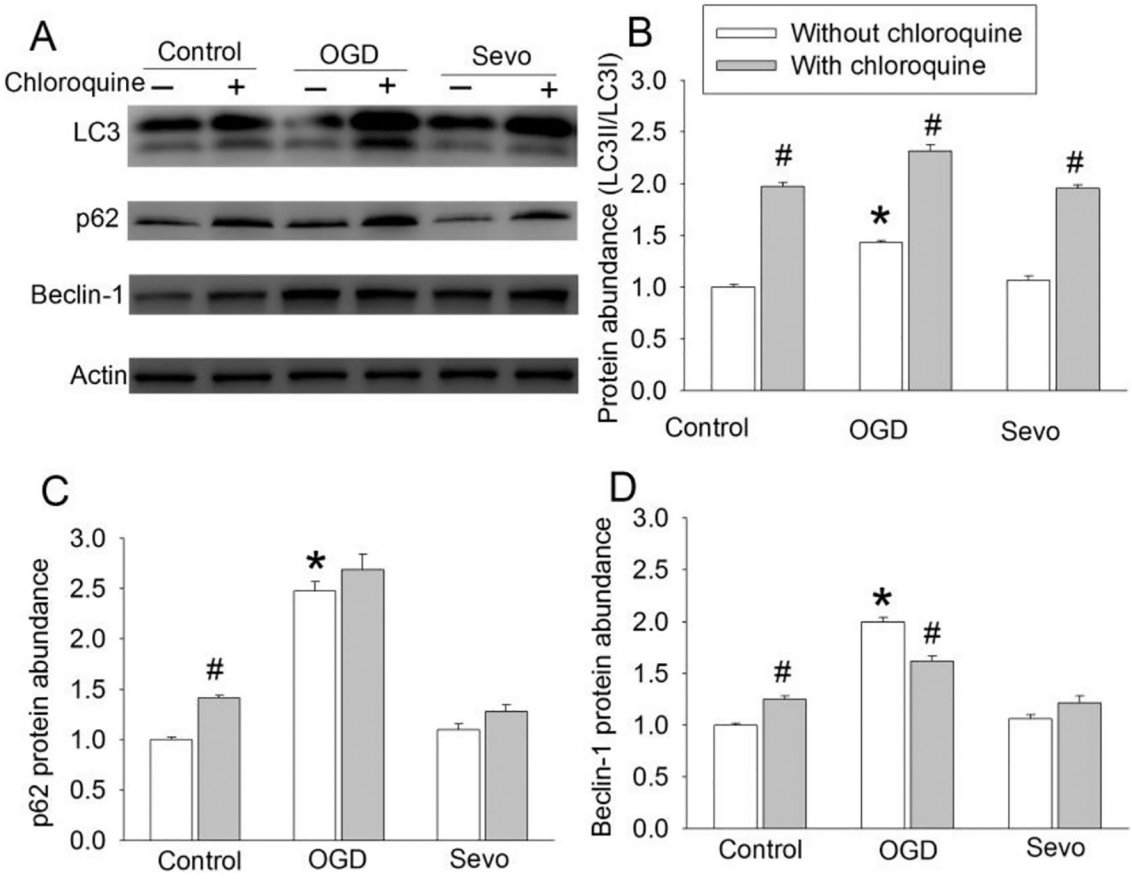

Fig. 3. Effects of 2% sevoflurane autophagic markers in human neuron-like cells.

Cells were exposed to 2% sevoflurane for 1 h and then harvested 19 h later for Western blotting. A: Representative images of Western blotting. B: Quantitative results of the ratio of LC3II to LC3I. C: Quantitative results of p62. D: Quantitative results of beclin-1. Results are means ± S.E.M. (n = 8). * $P < 0.05$ compared to control. # $P < 0.05$ compared with the corresponding condition without chloroquine. OGD: oxygen-glucose deprivation; Sevo: 2% sevoflurane.

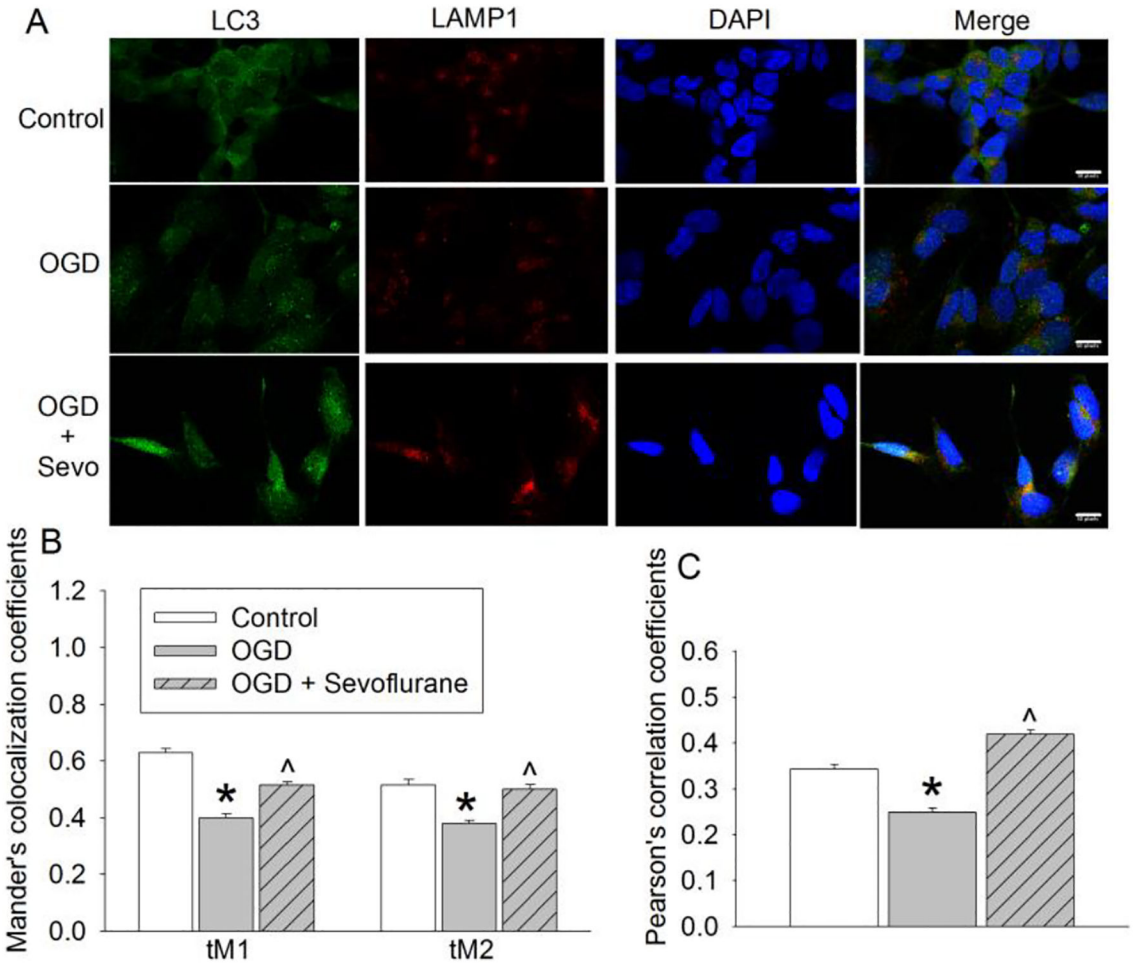

Fig. 4. Sevoflurane improved autophagosome-lysosome fusion.

Human neuron-like cells were subjected to 1 h OGD followed with 20 h simulated reperfusion. Cells were exposed to 2% sevoflurane for 1 h immediately after the onset of simulated reperfusion. A: Representative confocal microscopic images. Scale bar = 10 μm. B: Mander's co-localization coefficients. C: Pearson's correlation coefficients. Results are means ± S.E.M. (n = 150 cells). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD only. OGD: oxygen-glucose deprivation

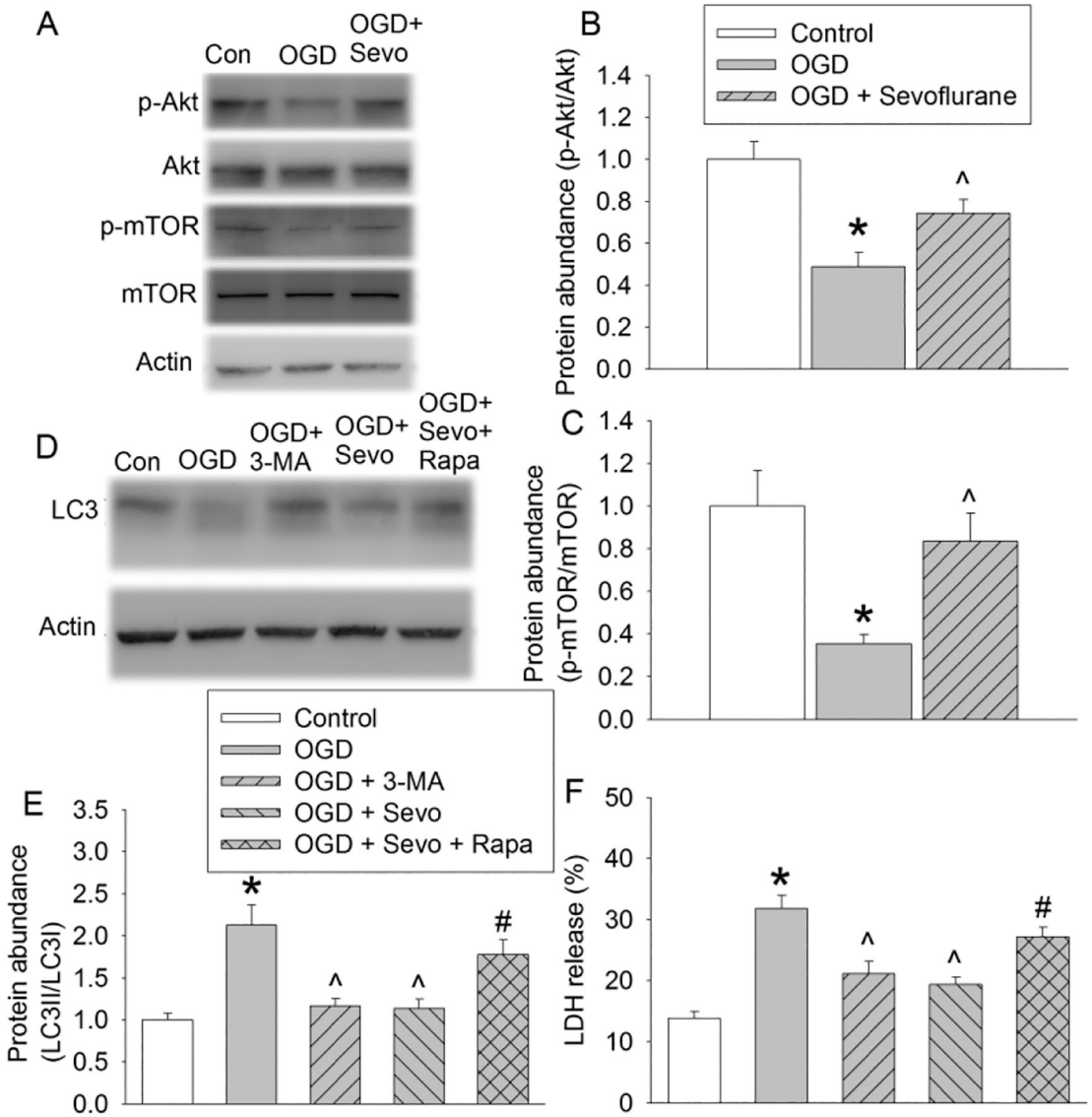

Fig. 5. Role of Akt-mTOR signaling in sevoflurane postconditioning-induced decrease of autophagy and cell protection.

Human neuron-like cells were subjected to 1 h OGD followed with 20 h simulated reperfusion. Cells were exposed to 2% sevoflurane for 1 h immediately after the onset of simulated reperfusion. 3-MA was added to cells 1 h prior to OGD. Rapamycin was added to cells 24 h prior to OGD. They were present until cells were harvested for assay. A: Representative images of Western blotting. B: Quantitative results of phospho-Akt. C: Quantitative results of phospho-mTOR. D: Representative images of Western blotting. E: Quantitative results of the ratio of LC3II to LC3I. F: LDH release. Results are means ± S.E.M. (n = 8 for panels B and C, = 10 for panel E, = 9 for panel F). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD group. # $P < 0.05$ compared with OGD plus sevoflurane postconditioning. Con: control; OGD: oxygen-glucose deprivation; 3-MA: 3-methyladenine; Sevo: 2% sevoflurane; Rapa: rapamycin.

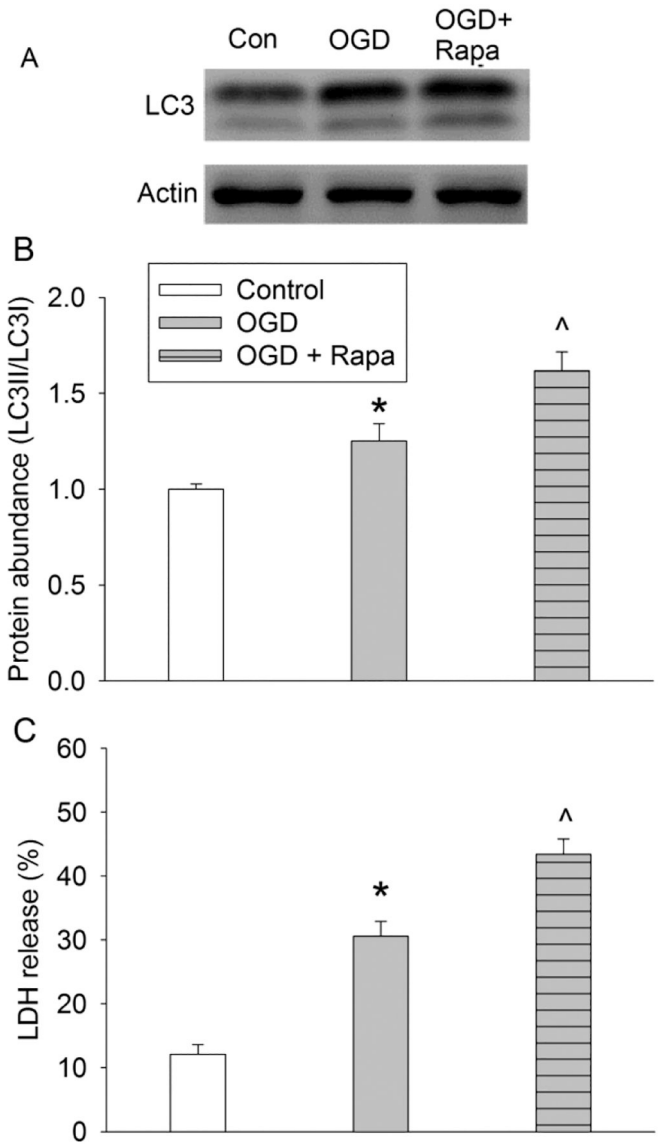

Fig. 6. Effects of mTOR inhibition on autophagosome formation and cell injury.

Human neuron-like cells were subjected to 1 h OGD followed with 20 h simulated reperfusion. Rapamycin was added to cells 24 h prior to OGD and was present until cells were harvested for assay. A: Representative images of Western blotting. B: Quantitative results of the ratio of LC3II to LC3I. C: LDH release. Results are means ± S.E.M. (n = 8 for panels B, and = 5 for panel C). * $P < 0.05$ compared to control. ^ $P < 0.05$ compared to OGD group. Con: control; OGD: oxygen-glucose deprivation; Rapa: rapamycin.