Lycopene prevents oxygen-glucose deprivation-induced autophagic death in SH-SY5Y cells via inhibition of the oxidative stress-activated AMPK/mTOR pathway

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Abstract. Lycopene has been reported to exert a protective effect on the brain against transient ischemia-induced damage; however, whether it could regulate autophagic neuronal death remains elusive. The present study aimed to investigate the role of autophagy in the protective effects of lycopene against neuronal damage and its underlying mechanism. Oxygen-glucose deprivation (OGD) was used to simulate neuronal ischemic injury in human SH-SY5Y cells. Lactate dehydrogenase (LDH) release assay revealed that OGD induced SH-SY5Y cell death. Western blotting demonstrated that OGD upregulated the expression levels of the autophagy marker proteins autophagy protein 5 (ATG5) and LC3II, but downregulated the autophagy substrate p62 in a time-dependent manner. By contrast, OGD-induced cell death was significantly inhibited by the autophagy inhibitors 3-methyladenine or bafilomycin A1 or by knockdown of ATG5, indicating that OGD may induce autophagic death in SH-SY5Y cells. Notably, lycopene was shown not only to prevent OGD-induced SH-SY5Y cell death, but was also able to effectively inhibit OGD-induced upregulation of ATG5 and LC3II, and downregulation of p62 in a dose-dependent manner. Mechanistically, it was suggested that lycopene inhibited OGD-induced activation of the AMPK/mTOR pathway via attenuation of oxidative stress by maintaining the intracellular antioxidant glutathione (GSH). Furthermore, the inhibitory role of lycopene in GSH depletion was found to be associated with the prevention of OGD-induced depletion of intracellular cysteine and downregulation of xCT. Collectively, the present study demonstrated that lycopene protected SH-SY5Y cells against OGD-induced autophagic death by inhibiting oxidative stress-dependent activation of the AMPK/mTOR pathway.

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Abbreviations: OGD, oxygen-glucose deprivation; LDH, lactate dehydrogenase; MDA, malondialdehyde; ER, endoplasmic reticulum

Key words: lycopene, autophagy, oxidative stress, OGD, xCT

Introduction

Transient global cerebral ischemia refers to a pathological deprivation of oxygen and glucose in the brain within a short time period, which can be caused by cardiac arrest, cardiopulmonary bypass surgery and other situations (1). During the process of cerebral ischemia, the hippocampal neurons are vulnerable to injury and delayed neuronal death could be triggered when the cerebral blood supply is recovered (2,3). Numerous factors, such as calcium overload, oxidative stress and activation of apoptotic pathways, have been proposed to lead to ischemia/reperfusion-induced neuronal death (1-3). Furthermore, accumulating evidence has shown that autophagy may serve a crucial role in regulation of ischemia/reperfusion-induced neuronal death (4-7).

Autophagy degrades long-lived proteins or damaged organelles through the lysosomal pathway, which is different from the ubiquitin-proteasome system, which clears intracellular short-lived proteins (3). As a determinant of cell destiny, autophagy serves dual roles in the regulation of cell function; autophagy protects cells against the damage induced by interior or exterior stresses, whereas over-activated autophagy can lead to excessive auto-digestion of cellular constituents and the initiation of autophagic cell death (programmed cell death) (4). It has been reported that induction of autophagy may prevent epilepsy- or neurodegenerative disease-induced brain damage (4); however, accumulating evidence has demonstrated that autophagy may also contribute to hypoxic-ischemia- or head trauma-induced brain injury (5,6). Previous reports have suggested that suppression of autophagy could inhibit transient ischemia-induced brain injury, whereas other studies have revealed that induction of autophagy may alleviate transient ischemia-induced brain damage (7,8). Therefore, the role of autophagy in regulation of transient ischemia-induced brain damage remains controversial.

Lycopene is a lipid-soluble carotenoid compound with potent anti-oxidative capacity (9). Previous reports have demonstrated that transient ischemia-induced organ damage, such as heart, kidney, testis and liver damage, could be

prevented by lycopene treatment (10-13). Notably, lycopene has been reported to act as an effective neuroprotectant, given that it not only prevents brain damage induced by transient focal ischemia or global ischemia (14-16), but also alleviates neuronal injury induced by various neurotoxic compounds, including colchicine, methylmercury, rotenone, amyloid β, trimethyltin and 6-hydroxydopamine (17-22). In addition, it has been shown that lycopene may protect against cell damage through numerous pathways, such as inhibiting oxidative stress, suppressing inflammation and regulating iron metabolism (14,17,18,23). However, the role of lycopene in autophagy regulation remains elusive. Chen *et al* (24) reported that lycopene protected H9C2 cardiomyocytes against hypoxia and reoxygenation through activation of autophagy. By contrast, Zeng *et al* (25) demonstrated that lycopene prevented hyperglycemia-induced damage in endothelial progenitor cells via inhibition of autophagy. Insufficient cerebral blood supply-induced oxygen-glucose deprivation (OGD) is a known important pathological basis that can induce neuronal death, and a common feature of focal ischemia and global ischemia (26). The human neuroblastoma SH-SY5Y cells used in the present study have similar characteristics to neurons, including morphology, neurochemistry and electrophysiology (26). The present study used an SH-SY5Y cell model of OGD to simulate cerebral ischemia, and investigated the role of autophagy in the protective effects of lycopene against neuronal damage and its underlying mechanism.

Materials and methods

Reagents and antibodies. Primary antibodies against AMPK (cat. no. ab32047), phosphorylated (p)-AMPK (cat. no. ab92701), LC3B (cat. no. ab192890), autophagy protein 5 (ATG5; cat. no. ab108327), p62 (cat. no. ab109012), mTOR (cat. no. ab2732), p-mTOR (cat. no. ab109268), xCT (cat. no. ab175186) and β-actin (cat. no. ab8226) were purchased from Abcam. 3-Methyladenine (3MA), bafilomycin A1 and lycopene were all purchased from Sigma-Aldrich; Merck KGaA. Lycopene was dissolved in tetrahydrofuran (THF) before each experiment. Other reagents were all purchased from Sigma-Aldrich; Merck KGaA, unless otherwise specified.

Cell culture and OGD. The human neuroblastoma SH-SY5Y cell line was obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and was verified using short tandem repeat analysis. Cells were cultured at 37˚C in an atmosphere containing 5% CO_2 in DMEM (Sigma-Aldrich; Merck KGaA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l glutamine (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was replaced twice a week. OGD was induced according to a previously published protocol (3). The cells underwent OGD for 3, 6, 12 and 24 h, or were pretreated for 1 h at 37˚C with target chemicals 3MA (5 mmol/l), bafilomycin A1 (1.5 μmol/l) and GSH (10 mmol/l; Sigma-Aldrich; Merck KGaA) and lycopene (0.5, 2.0 and 8.0 μmol/l), and then underwent OGD at 37˚C for 24 h. Moreover, the cells were treated with scrambled small interfering RNA (siRNA), ATG5 siRNA or AMPK siRNA prior to undergoing OGD at 37˚C for 24 h.

Lactate dehydrogenase (LDH) release cell death assay. Elevated LDH was used to detect cell death caused by cellular membrane damage using a detection kit (Beyotime Institute of Biotechnology). Control cells were treated with the lycopene solvent THF (0.1%), and the other groups of cells were treated with lycopene at 0.5, 2.0 and 8 μmol/l prior to OGD at 37˚C for 24 h. LDH release was detected according to the manufacture's protocol. The absorbance value of each sample was read at 490 nm, as specified by the manufacturer's protocol. The death ratio was calculated using the following formula: Cell death ratio (%) = (A sample-A control/A max-A control) x100. A sample refers to the sample absorbance value; A control refers to the absorbance value of the control group; A max refers to the absorbance value of the positive group (which consisted of cells treated with Triton X-100).

Measurement of total intracellular GSH. The DTNB-GSSH reductase recycling assay kit (Beyotime Institute of Biotechnology) was used to detect total intracellular GSH according to the manufacturer's instructions. Briefly, the collected cells ($1x10^7$) were treated with protein-removing buffer S, homogenized on ice with a homogenizer and centrifuged at 10,000 x g for 10 min at 4˚C, in order to obtain the supernatant used to assess intracellular total GSH. GSH content was detected at an absorbance value of 412 nm and was expressed as a ratio to the absorbance value of the control cells. GSH was purchased from Sigma-Aldrich; Merck KGaA.

Measurement of total intracellular cysteine. A cysteine assay kit (Nanjing Jiancheng Bioengineering Institute) was used to detect intracellular cysteine. The experiment was performed according to the manufacturer's instructions. Briefly, the collected cells ($1x10^7$) were treated with reagent A, homogenized on ice and centrifuged at 8,000 x g for 4 min at 4˚C, in order to obtain the supernatant used to assess cysteine levels. After the protein concentration of the supernatant was measured using the Pierce BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), a 20-μl sample was incubated with 100 μl reagent B and 100 μl reagent C for 15 min at room temperature and read at an absorbance of 600 nm using a microplate reader. Finally, the results were expressed as a ratio to the absorbance value of the control cells.

Measurement of intracellular reactive oxygen species (ROS). Intracellular ROS levels were detected using the ROS probe DCFH-DA (Beyotime Institute of Biotechnology). Firstly, SH-SY5Y cells ($1x10^6$) were seeded onto 96-well plates and after 24 h, OGD was performed at 3, 6, 12 and 24 h. Secondly, the cells were washed in PBS twice and then stained with 20 μmol/l DCFH-DA for 30 min in the dark at 37˚C. Thirdly, these cells were dissolved with 1% Triton X-100, followed by fluorescence detection using a spectrometer (HTS 7000; Perkin Elmer, Inc.). The excitation wavelength was 485 nm and the emission wavelength was 530 nm. Furthermore, SH-SY5Y cells ($3x10^6$) seeded onto a 3-cm dish were observed under a fluorescence microscope (Olympus IX71; Olympus Corporation). This group of cells were also treated with OGD and stained with 20 μmol/l DCFH-DA as aforementioned.

Measurement of intracellular malondialdehyde (MDA). An MDA assay kit (Nanjing Jiancheng Bioengineering Institute)

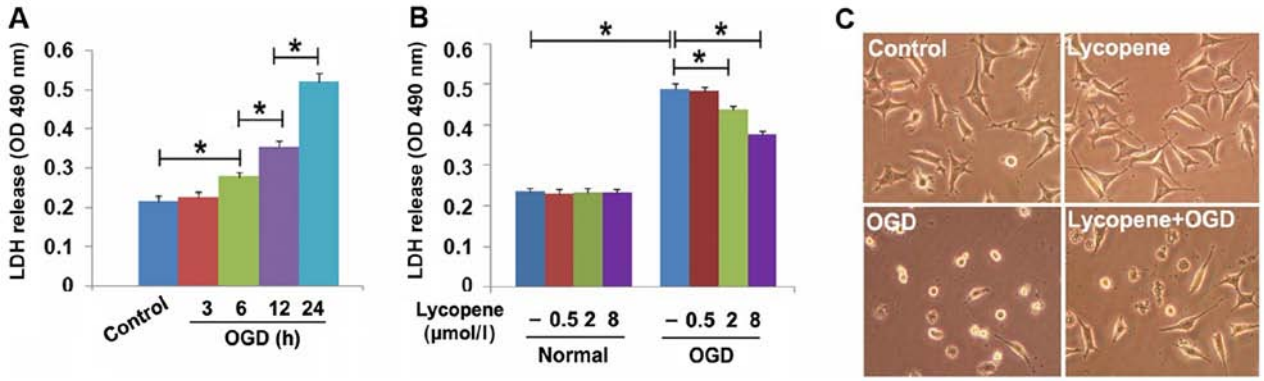

Figure 1. Lycopene prevents OGD-induced autophagic death in SH-SY5Y cells. (A) LDH release assay revealed that OGD induced SH-SY5Y cell death in a time-dependent manner. (B) Lycopene inhibited OGD-induced SH-SY5Y cell death. (C) Representative images acquired from light microscopy (magnification, x20) depicted OGD-induced morphological changes in cells with or without lycopene treatment. *P<0.01. LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation.

was used to detect intracellular MDA levels. The experiment was performed according to the manufacturer's instructions. Briefly, the collected cells ($1x10^7$) were added to RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology), homogenized on ice and centrifuged at 1,600 x g for 10 min at 4˚C to obtain the supernatant. Once the protein concentration of the supernatant was measured using the Pierce BCA protein assay kit, a 100-μl sample was incubated with 100 μl test solution for 15 min at 100˚C. Once the samples were cooled to room temperature, they were centrifuged at 1,000 x g for 10 min at 37˚C to obtain the supernatant, the absorbance of which was measured at 530 nm using a microplate reader. MDA content was expressed as a ratio to the absorbance value of the control cells.

Transfection of siRNA and GFP-LC3 lentiviral vectors. SH-SY5Y cells ($2x10^5$) were seeded onto 10-cm dishes. The siRNAs were transfected into the cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (Opti-MEM:Lipofecta-mine 3000:siRNA, 100:1:5; concentration of siRNA transfected, 20 ng/μl). Cells were then treated with OGD after overnight siRNA transfection. The siRNAs used in the present study were purchased from Shanghai GenePharma Co., Ltd. and are listed as follows: ATG5 siRNA, 5'-3'):GACGUUGGUAACUGACAAATT; AMPK siRNA, 5'-3'):GCGUGUACGAAGGAAGAAUTT; scrambled siRNA, 5'-3'):UUCUCCGAACGUGUCACGUTT-3'.

For GFP-LC3 lentiviral vector transfection, SH-SY5Y cells ($1x10^6$) were seeded onto 6-well plates and cultured for 18 h, followed by infection with GFP-LC3 lentiviral vectors (Shanghai GeneChem Co., Ltd.) for 12 h (MOI, 2.5). After being incubated in normal culture medium for 48 h and undergoing OGD, the cells were observed by confocal microscopy (FV10i; Olympus Corporation).

Gel electrophoresis and western blotting. SH-SY5Y cells were lysed with ice-cold cellular membrane lysis buffer (Beyotime Institute of Biotechnology) and homogenized with a glass Pyrex microhomogenizer (20 strokes). The protein content of the supernatant was obtained after the homogenates were centrifuged at 1,000 x g for 10 min at 4˚C; protein concentration was detected using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.).

Equal amounts of protein (20 μg) were separated by SDS-PAGE on 7% gels and transferred to PVDF membranes. After blocking with 3% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, PVDF membranes were immunoblotted with primary antibodies against AMPK (1:1,000), p-AMPK (1:1,000), LC3 (1:1,000), ATG5 (1:1,500), p62 (1:1,000), xCT (1:1,000), mTOR (1:1,000) and p-mTOR (1:1,000) overnight at 4˚C. β-actin (1:1,000) was used as a loading control. The membranes were then washed three times in PBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies: Goat anti-rabbit IgG (cat. no. ab6721) and horse anti-mouse IgG (cat. no. ab6789) (1:2,000 dilution; Abcam) at room temperature for 2 h. Finally, blots were washed three times. The immunoreactive proteins were visualized using an ECL kit (cat. no. P0018F; Beyotime Institute of Biotechnology) and analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

Light microscopy. The SH-SY5Y cells ($1x10^7$/well) were seeded on a 6-well plate and underwent OGD at 37˚C for 24 h in the presence or absence of lycopene (8 μmol/l). Subsequently, morphological changes of the cells were observed under a light microscope (Olympus BX51; Olympus Corporation). Representative images were obtained by a researcher who was blinded to the cell groups.

Statistical analysis. SPSS statistical software (version 19.0; IBM Corp.) was utilized for data analysis. All data shown in the present study represent at least four independent experiments and are presented as the mean ± SEM. Data were statistically analyzed using one-way or two-way ANOVA, followed by LSD post hoc test if there were less than four groups assessed or Tukey's post hoc method if there were more than three groups assessed. P<0.05 was considered to indicate a statistically significant difference.

Results

Lycopene inhibits OGD-induced SH-SY5Y cell death. Since the LDH release assay is a common method used to evaluate cell death, the present study used this method to detect the effects of lycopene on OGD-induced SH-SY5Y cell death. As shown in Fig. 1A, OGD significantly induced SH-SY5Y

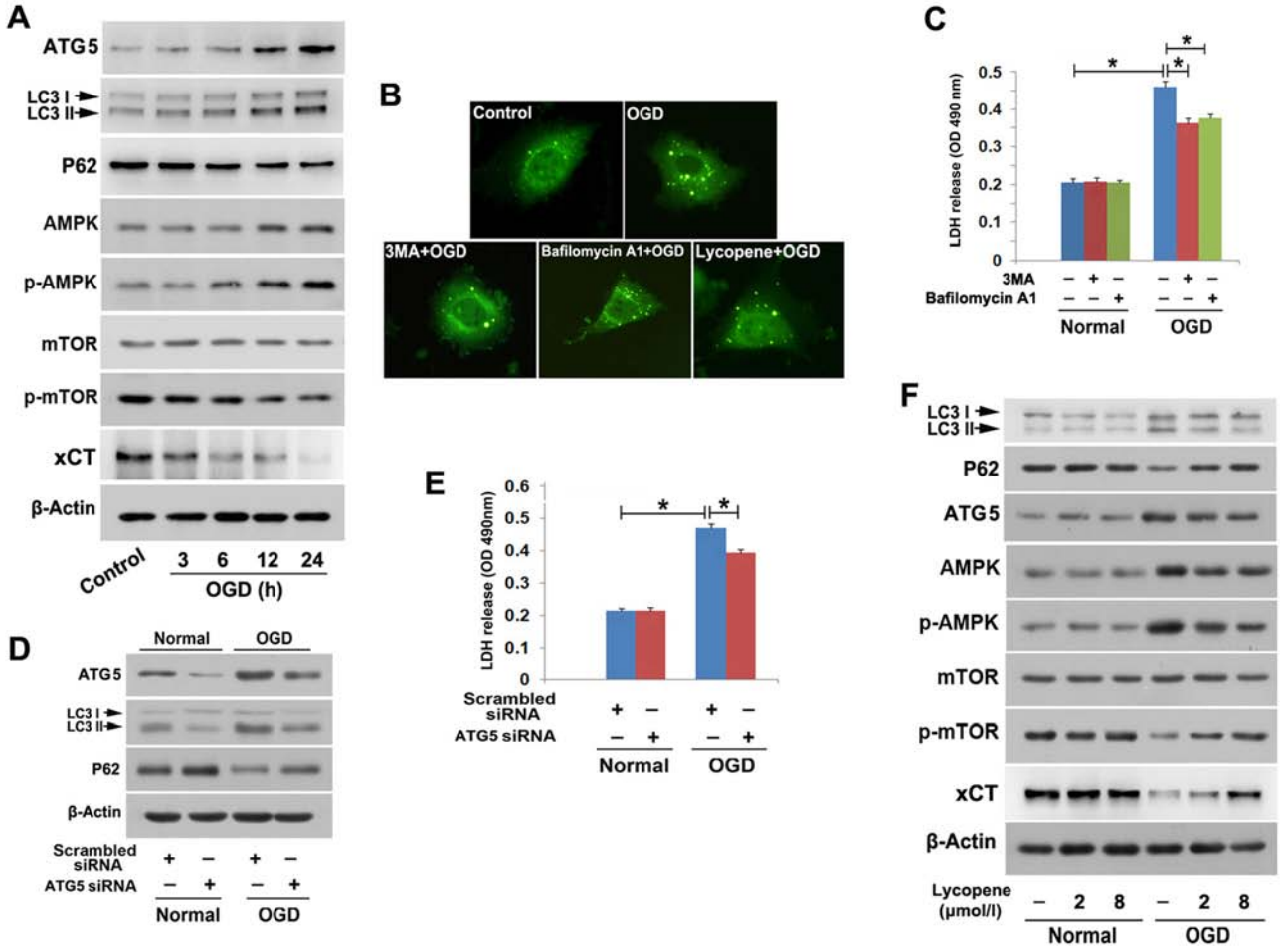

Figure 2. Lycopene inhibits OGD-induced autophagy. (A) Western blotting proved that OGD increased the expression levels of ATG5 and LC3II, and decreased the expression levels of p62 in a time-dependent manner. Moreover, OGD induced upregulation of AMPK, p-AMPK and p-mTOR, but downregulated xCT. (B) Laser scanning microscopy (magnification, x40) showed that OGD-induced formation of green puncta in cells transfected with lentivirus-GFP-LC3 was inhibited by pretreatment with 3MA or lycopene, but reinforced by bafilomycin A1. (C) LDH release assay demonstrated that both 3MA and bafilomycin A1 inhibited OGD-induced SH-SY5Y cell death. (D) Western blotting revealed that knockdown of ATG5 with siRNA attenuated OGD-induced upregulation of LC3II and downregulation of p62. (E) LDH release assay revealed that knockdown of ATG5 with siRNA prevented OGD-induced SH-SY5Y cell death. (F) Lycopene pretreatment obviously reversed OGD-induced changes in LC3II, p62, ATG5, AMPK, p-AMPK, p-mTOR and xCT expression. LDH release data were presented as the mean ± SEM (n=5 per group). *P<0.01. 3MA, 3-methyladenine; ATG5, autophagy protein 5; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; p, phosphorylated; siRNA, small interfering RNA.

cell death at 6 h, which was markedly increased when OGD treatment time was extended to 12 and 24 h, thus indicating that OGD induced SH-SY5Y cell death in a time-dependent manner. Subsequently, the cells were treated with 0.5, 2.0 and 8.0 μmol/l lycopene for 1 h, and then underwent OGD for 24 h. LDH release assay revealed that OGD-induced SH-SY5Y cell death was prevented by 2 μmol/l lycopene, and this prevention was more apparent when the dosage of lycopene was increased to 8 μmol/l (Fig. 1B). Moreover, light microscopy revealed that most of the cells that underwent OGD became shrunken and exhibited a round shape compared with the cells in the control group, which exhibited a polygonal shape. By contrast, pretreatment with 8 μmol/l lycopene inhibited OGD-induced morphological changes in SH-SY5Y cells (Fig. 1C). These results indicated that lycopene may protect against the lethal effects of OGD on SH-SY5Y cells.

Lycopene inhibits OGD-induced autophagic death of SH-SY5Y cells. Induction of lethal autophagy has been reported to be a pathway accounting for OGD-triggered death in SH-SY5Y cells (8). To elucidate the mechanism underlying the protective effect of lycopene on OGD-induced SH-SY5Y cell death, the present study examined the effect of lycopene on OGD-induced autophagy.

Firstly, OGD-induced changes in the expression levels of autophagy marker proteins were detected through western blotting. OGD upregulated the protein expression levels of ATG5 and LC3II (autophagy marker proteins), and downregulated the expression levels of p62 (substrate of autophagy) in a time-dependent manner (Fig. 2A). To determine whether OGD induced formation of autophagosomes, the GFP-LC3 lentiviral vector was transfected into SH-SY5Y cells, which then did or did not undergo OGD. As revealed by laser scanning confocal microscopy, numerous green puncta were induced to form in the cytoplasm by OGD when compared with the control cells (Fig. 2B). Subsequently, the cells were treated with 3MA and bafilomycin A1 prior to OGD treatment. As an inhibitor of activated PI3K vps34, 3MA effectively prevents autophagy initiation (4). Bafilomycin A1 inactivates autophagic flux by preventing the fusion of autophagosomes with lysosomes (4).

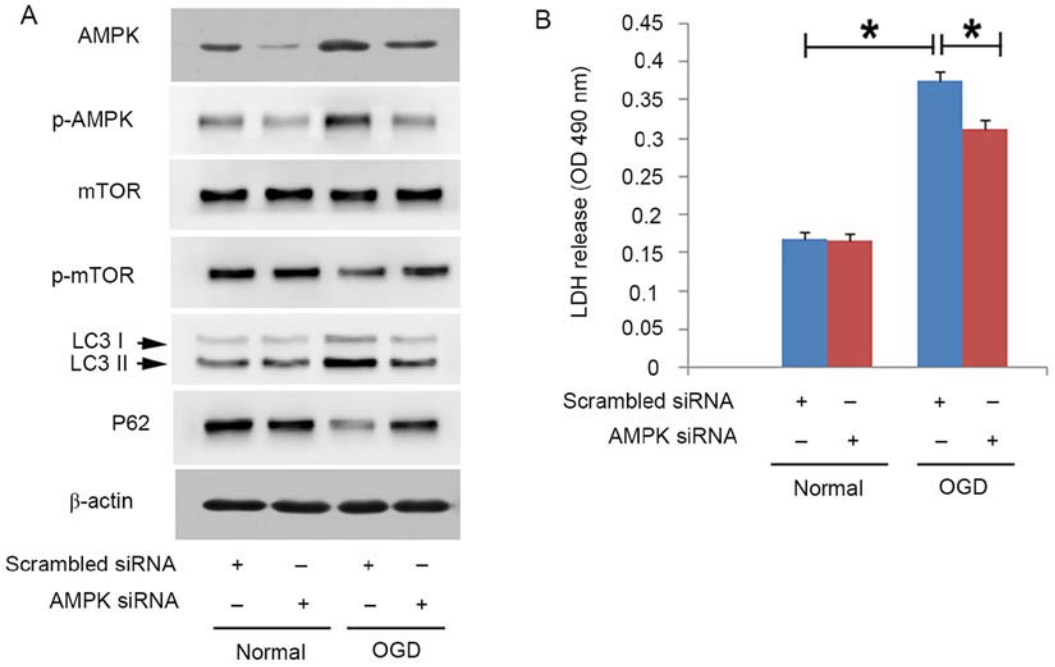

Figure 3. Lycopene prevents OGD-induced activation of AMPK. (A) Western blotting showed that knockdown of AMPK with siRNA inhibited OGD-induced upregulation of AMPK and p-AMPK, downregulation of p-mTOR and changes in autophagy-related marker proteins. (B) LDH release assay demonstrated that knockdown of AMPK with siRNA decreased OGD-induced SH-SY5Y cell death. Data are presented as the mean ± SEM (n=5 per group). *P<0.01. LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; p, phosphorylated; siRNA, small interfering RNA.

The results of the LDH release assay revealed that pretreatment with either 3MA (5 mmol/l) or bafilomycin A1 (1.5 μmol/l) for 1 h significantly prevented OGD-induced SH-SY5Y cell death (Fig. 2C). Moreover, laser scanning confocal microscopy demonstrated that OGD-induced formation of green puncta was decreased by pretreatment with 3MA, but increased in the presence of bafilomycin A1 (Fig. 2B). These findings indicated that OGD may induce activation of autophagic flux, which could contribute to OGD-induced SH-SY5Y cell death.

To further verify the role of autophagy in OGD-induced SH-SY5Y cell death, the present study introduced siRNA to knockdown ATG5 and examined OGD-induced SH-SY5Y cell death. The results demonstrated that knockdown of ATG5 not only reversed OGD-induced upregulation of LC3II and downregulation of p62, but also prevented OGD-induced SH-SY5Y cell death (Fig. 2D and E). Therefore, these results indicated that OGD may induce lethal autophagy in SH-SY5Y cells.

Notably, western blotting revealed that OGD-induced upregulation of ATG5 and LC3II, and downregulation of p62, were all inhibited following treatment with 2 μmol/l lycopene (Fig. 2F); the effects of lycopene were more apparent when the dosage was increased to 8 μmol/l. Meanwhile, laser scanning microscopy demonstrated that 8 μmol/l lycopene effectively inhibited OGD-induced formation of green puncta in the cytoplasm of SH-SY5Y cells (Fig. 2B), which was different from the effect of bafilomycin A1, but similar to the effect of 3MA. Collectively, these data suggested that lycopene may prevent OGD-induced lethal autophagy in SH-SY5Y cells through inhibition of autophagy initiation, not through blocking OGD-induced activation of autophagy flux.

Lycopene inhibits OGD-induced activation of the AMPK/mTOR pathway. Since AMPK, which senses dysfunctional energy metabolism, serves a key role in promoting autophagy occurrence (27), the present study assessed whether AMPK was involved in OGD-induced autophagic death in SH-SY5Y cells. As shown in Fig. 2A, OGD upregulated the protein expression levels of AMPK and p-AMPK (active form of AMPK) in SH-SY5Y cells, which became more apparent with the extension of OGD duration. Concomitantly, OGD decreased the expression levels of p-mTOR in a time-dependent manner, despite no obvious changes in mTOR at each indicated time point (Fig. 2A). In addition, knockdown of AMPK with siRNA not only prevented OGD-induced upregulation of p-AMPK, but also prevented the downregulation of p-mTOR (Fig. 3A). Since de-phosphorylation of mTOR is considered a downstream biochemical event of activated AMPK (3), it was hypothesized that OGD activated the AMPK/mTOR pathway in SH-SY5Y cells. Additionally, knockdown of AMPK using siRNA not only inhibited OGD-induced upregulation of LC3II and downregulation of p62 (Fig. 3A), but also significantly prevented OGD-induced SH-SY5Y cell death (Fig. 3B). Therefore, these results indicated that exposure to OGD may lead to autophagic death through activation of the AMPK/mTOR pathway in SH-SY5Y cells.

Notably, pretreatment with 2 or 8 μmol/l lycopene markedly decreased OGD-induced upregulation of p-AMPK and downregulation of p-mTOR (Fig. 2A). Moreover, the inhibitory effect of 8 μmol/l lycopene on OGD-induced activation of the AMPK/mTOR pathway was more obvious than 2 μmol/l lycopene. These findings demonstrated that lycopene inhibited OGD-induced activation of the AMPK/mTOR pathway and consequently prevented autophagic death.

Lycopene prevents OGD-induced oxidative stress. Given that oxidative stress is one of the important factors that activates

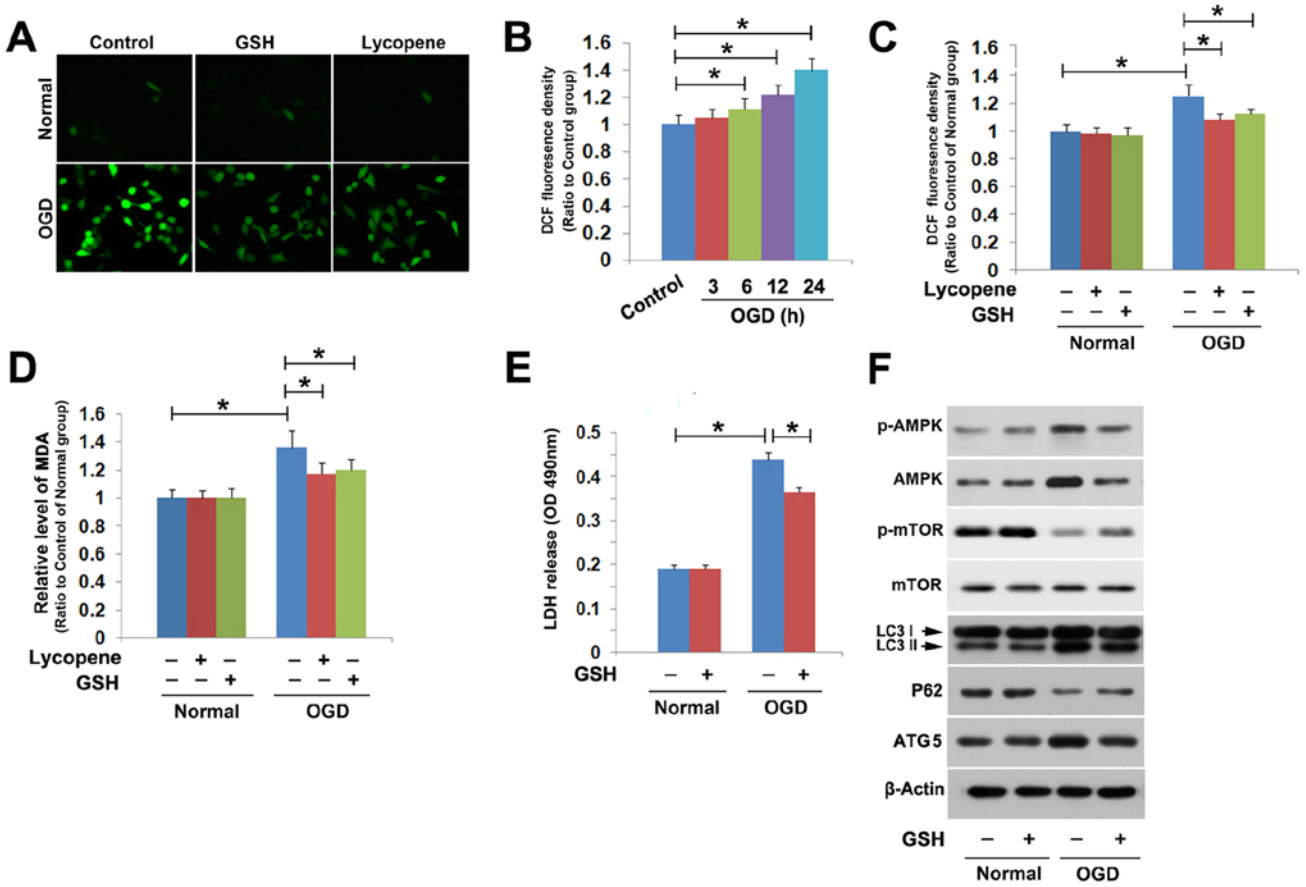

Figure 4. Lycopene prevents OGD-induced oxidative stress. (A) Representative images acquired from fluorescence microscopy (magnification, x20) showed that OGD markedly increased green fluorescence; this phenomenon was inhibited in the presence of GSH and lycopene. (B) Statistical analysis of the green fluorescence density showed that OGD induced overproduction of ROS in a time-dependent manner. (C) OGD-induced ROS was attenuated in the presence of GSH and lycopene. (D) Pretreatment with GSH or lycopene inhibited OGD-induced MDA. (E) LDH release assay depicted that GSH inhibited OGD-induced SH-SY5Y cell death. (F) Pretreatment with GSH prevented OGD-induced activation of AMPK, autophagy marker proteins and inhibition of p-mTOR. Data are presented as the mean ± SEM (n=5 per group). *P<0.01. ATG5, autophagy protein 5; GSH, glutathione; LDH, lactate dehydrogenase; MDA, malondialdehyde; OGD, oxygen-glucose deprivation; p, phosphorylated; ROS, reactive oxygen species.

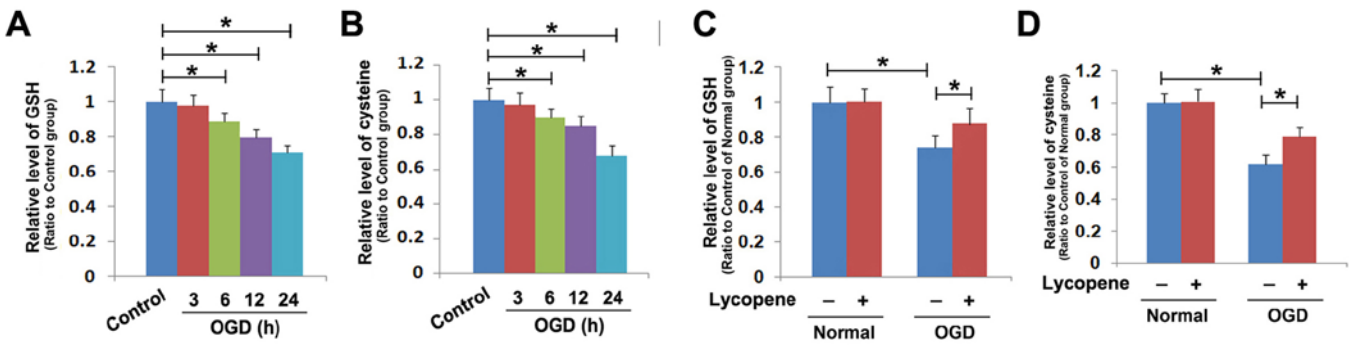

Figure 5. Lycopene inhibits OGD-induced depletion of GSH. (A) OGD decreased the levels of GSH in a time-dependent manner. (B) OGD decreased the levels of cysteine in a time-dependent manner. (C) Lycopene reversed OGD-induced GSH depletion. (D) Lycopene prevented OGD-induced depletion of cysteine. Data are presented as the mean ± SEM (n=5 per group). *P<0.01. GSH, glutathione; OGD, oxygen-glucose deprivation.

the AMPK/mTOR pathway in SH-SY5Y cells (28), the present study assessed whether lycopene could inhibit OGD-induce oxidative stress in SH-SY5Y cells.

The ROS probe DCFH-DA was used to detect intracellular ROS. Fluorescence microcopy revealed that green fluorescence (ROS) was much brighter in the cells stressed with OGD for 24 h compared with that in the control group (Fig. 4A). Statistical analysis of the fluorescence density revealed that OGD significantly increased intracellular ROS levels at 6 h, and higher levels were detected at 12 and 24 h (Fig. 4B). However, pretreatment with the antioxidant GSH (10 mmol/l) for 1 h significantly mitigated OGD-induced increases in ROS levels (Fig. 4A and C). In addition, the levels of OGD-induced MDA, which is a product of lipid oxidation, were reduced by

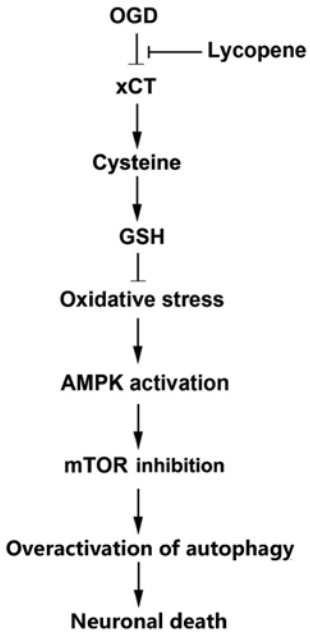

Figure 6. Schematic model of the inhibitory effect of lycopene on OGD-induced autophagic death. GSH, glutathione; OGD, oxygen-glucose deprivation.

GSH (Fig. 4D). GSH pretreatment also inhibited OGD-induced SH-SY5Y cell death (Fig. 4E). Therefore, these results indicated that OGD induced SH-SY5Y cell death through the oxidative stress pathway.

Western blotting revealed that mitigation of intracellular ROS with GSH effectively prevented OGD-induced phosphorylation of AMPK and dephosphorylation of mTOR (Fig. 4F). Furthermore, it was revealed that OGD-induced upregulation of ATG5 and LC3II, and downregulation of p62, was also reversed in the presence of GSH (Fig. 4F). These findings indicated that oxidative stress may be required for OGD-induced activation of the AMPK/mTOR pathway and subsequent lethal autophagy in SH-SY5Y cells.

Notably, pretreatment with 8 μmol/l lycopene for 1 h effectively attenuated OGD-induced increases in ROS and MDA (Fig. 4A, C and D), indicating that lycopene may inhibit the oxidative stress caused by OGD. Collectively, these results suggested that lycopene inhibited OGD-induced activation of the AMPK/mTOR pathway and lethal autophagy through mitigation of oxidative stress.

Lycopene inhibits OGD-induced depletion of intracellular antioxidant GSH. Given that GSH is an interior antioxidant within cells, in order to uncover the mechanism underlying the inhibitory effects of lycopene on OGD-induced oxidative stress in SH-SY5Y cells, OGD-induced GSH levels were compared between the cells pretreated with or without lycopene. As shown in Fig. 5A, OGD decreased the intracellular levels of GSH in SH-SY5Y cells at 6 h, which was exacerbated when the exposure time to OGD was extended to 12 and 24 h. Cysteine that is transformed from cystine is a material used for GSH synthesis (29), the present study thus tested whether OGD could deplete intracellular cysteine. The results revealed that exposure to OGD significantly reduced cysteine in SH-SY5Y cells at 6 h, which was aggravated at 12 and 24 h (Fig. 5B). These findings indicated that OGD inhibited the levels of intracellular GSH and cysteine in a time-dependent manner. However, the OGD-induced decrease in GSH levels was prevented by treatment with 8 μmol/l lycopene for 1 h (Fig. 5C). Moreover, pretreatment with lycopene markedly prevented the OGD-induced reduction in cysteine (Fig. 5D). These findings indicated that the inhibitory effect of lycopene on OGD-induced depletion of GSH may be associated with maintaining intracellular levels of cysteine.

Cystine, which could be used to generate cysteine, is transported into cells through the cystine/glutamate antiporter (29). The present study further examined the protein expression levels of xCT (also known as SLC7A11), which is a specific light-chain subunit of the cystine/glutamate antiporter, in the cells that did or did not undergo OGD by western blotting. It was revealed that OGD downregulated the protein expression levels of xCT in a time-dependent manner (Fig. 2A). However, the downregulation of xCT caused by OGD was markedly prevented in the cells pretreated with 2 μmol/l lycopene for 1 h; the downregulation was further inhibited when the dosage of lycopene was increased to 8 μmol/l (Fig. 2F). There results demonstrated that lycopene inhibited OGD-induced downregulation of xCT, which may account for the inhibitory effect of lycopene on OGD-induced depletion of cysteine and GSH.

Discussion

The present study provided evidence that OGD induced autophagic death in SH-SY5Y cells, which was accompanied by a time-dependent upregulation of the autophagy marker proteins ATG5 and LC3II, and downregulation of the autophagy substrate p62. Conversely, OGD-induced cell death was inhibited when ATG5 was knocked down with siRNA, or in the presence of autophagy inhibitors 3MA or bafilomycin A1. Notably, it was demonstrated that lycopene not only prevented OGD-induced SH-SY5Y cell death, but also effectively inhibited OGD-induced changes in the protein expression levels of ATG5, LC3 and p62 in a dosage-dependent manner. Mechanistically, lycopene inhibited OGD-induced activation of the AMPK/mTOR pathway via mitigation of oxidative stress by preventing the depletion of the intracellular antioxidant GSH. Further studies revealed that lycopene inhibited the OGD-induced decrease in cysteine and attenuated the downregulation of xCT. Collectively, these data demonstrated that lycopene prevented OGD-induced lethal autophagy via maintaining intracellular GSH levels in SH-SY5Y cells (Fig. 6).

Autophagy is known to be an evolutionarily conserved and highly regulated homeostatic process by which cells acquire nutrients through degradation of cytoplasmic macromolecules and organelles using the lysosomal system (30). Overactivated autophagy leads to cell death, which is named autophagic death or type II programmed death. Similar to apoptosis, autophagy is one of the crucial factors leading to SH-SY5Y cell death under the OGD condition, which is commonly used as an ischemic model *in vitro* (31,32). The present study revealed that OGD-induced upregulation of LC3II was attenuated by 3MA, which could inhibit autophagy initiation, but was enhanced by bafilomycin A1, which could block combination of autophasomes with lysosomes. Consistently, OGD-induced

formation of green puncta in the cells transfected with LC3 was decreased by 3MA, whereas it was strengthened by bafilomycin A1. Thus, these results indicated that OGD activated autophagy in SH-SY5Y cells. It was previously reported that several compounds, such as picroside II (31) and cornin (32), could protect SH-SY5Y cells against OGD-induced death by weakening overactivated autophagy. Therefore, targeting autophagy is thought to be a strategy to prevent OGD-induced damage. Although accumulating evidence has revealed that lycopene may inhibit neuronal apoptosis induced by amyloid-β, subarachnoid hemorrhage and cerebral ischemia, the role of lycopene in autophagy remains unclear (16,33,34). In contrast to a previous report, which revealed that lycopene protected H9C2 cardiomyocytes against hypoxia/reoxygenation-induced apoptosis through increased protective autophagy (24), the present study demonstrated that pretreatment with lycopene not only inhibited OGD-induced upregulation of ATG5 and LC3II, and downregulation of p62, but also attenuated OGD-induced SH-SY5Y cell death. Thus, these data indicated that lycopene inhibited OGD-triggered neuronal death via preventing the activation of lethal autophagy.

The AMPK/mTOR signaling pathway is well established as an important regulator in the pathological process of neuronal damage caused by head trauma and cerebral ischemia (35,36). Moreover, it has been reported to be involved in autophagic death of various cell types, such as cardiomyocytes, vascular endothelial cells and renal tubular cells (37-39). Thus, it was hypothesized that autophagic neuronal death could be rescued through inhibition of the AMPK/mTOR signaling pathway. Li *et al* (37) demonstrated that thioredoxin-2 attenuated OGD-induced autophagic death in H9c2 cardiomyocytes via suppression of AMPK/mTOR signaling. Sun *et al* (40) reported that propofol protected neurons against OGD-induced lethal autophagy via inhibition of the AMPK/mTOR pathway. Similarly, the present study demonstrated that lycopene effectively reversed OGD-induced phosphorylation of AMPK and de-phosphorylation of mTOR, indicating that the preventive effects of lycopene against OGD-induced lethal autophagy in SH-SY5Y cells were associated with inhibition of the AMPK/mTOR pathway. In addition to the AMPK/mTOR pathway, activated JNK and HIF1-α have been reported to contribute to OGD-induced lethal autophagy (31,41). Notably, previous studies have shown that lycopene could suppress the activation of JNK, as well as inhibit the activity of HIF1-α (31,42). Therefore, lycopene may inhibit OGD-induced lethal autophagy via multiple pathways.

Oxidative stress is a crucial factor leading to AMPK/mTOR activation, and it is characterized by intracellular accumulation of ROS resulting from a disrupted equilibrium between ROS generation and clearance (28). It was reported previously that ROS not only upregulated the protein expression levels of AMPK, but also promoted phosphorylation of AMPK. Similarly, the present study revealed that OGD not only induced upregulation of both AMPK and p-AMPK, but also promoted intracellular levels of ROS. Lycopene has been shown to act as a potent antioxidant; previous studies have demonstrated that lycopene prevented neuronal death induced by ischemia/reperfusion, colchicine, methylmercury, rotenone and amyloid β through inhibition of oxidative stress (16-20). Moreover, it was previously reported that lycopene significantly inhibited hydrogen peroxide (H_2O_2)-induced autophagic SH-SY5Y cell death (43). The present study revealed that lycopene markedly suppressed OGD-induced intracellular accumulation of ROS and MDA, indicating that lycopene inhibited OGD-induced AMPK/mTOR activation via inhibition of oxidative stress. Previous studies have reported that lycopene may serve anti-oxidative roles through numerous mechanisms, such as inhibition of mitochondrial superoxide generation, activation of the Nrf2/HO-1 signaling pathway, inhibition of the nitric oxide pathway, and maintaining the protein levels and activity of catalase that could degrade H_2O_2 (13,15,17,43). Moreover, it has also been shown that lycopene could inhibit H2O2-induced apoptosis of SH-SY5Y cells via inhibition of oxidative stress-activated caspase-3 and nuclear translocation of AIF (43). By contrast, the present study demonstrated that lycopene inhibited OGD-induced SH-SY5Y cell death via inhibition of excessive activation of autophagy. Apoptosis is morphologically characterized by cell membrane blebbing, chromatin condensation and formation of apoptotic bodies (43); however, autophagy is characterized by the cytoplasmic formation of numerous vacuoles containing cytosolic components or organelles (35,36). At molecular levels, autophagy activation is associated with ATG5 and ATG12, whereas apoptosis is related to cascade activation of caspase-9 and caspase-3 (35,36,43). Thus, apoptosis and autophagic death are two different types of programmed cell death, despite the fact that they can both be induced under the condition of oxidative stress. Therefore, the novelty of the present study is that lycopene exerted a protective effect on SH-SY5Y cells via inhibition of autophagy.

GSH is an intracellular antioxidant, which can be used by glutathione peroxidase 4 to reduce intracellular ROS and lipid oxidation products (29). The present study revealed that OGD-induced depletion of GSH was reversed in the presence of lycopene. Furthermore, cystine can be converted into cysteine, which can then be used as material for GSH synthesis, and as a specific light-chain subunit of the cystine/glutamate antiporter, xCT is required for transporting extracellular cystine into cells (29). The present results demonstrated that lycopene inhibited OGD-induced decreases in cysteine and downregulation of xCT (SLC7A11). Thus, it was suggested that the inhibitory role of lycopene in OGD-induced oxidative stress was associated with maintaining GSH levels by preventing OGD-induced dysfunction of the cystine/glutamate antiporter.

Endoplasmic reticulum (ER) stress could be activated by intracellular ROS and has been suggested as a key regulator of autophagy (44). Furthermore, in previous studies, ER stress not only promoted H_2O_2-induced autophagic death, but also contributed to OGD-induced intracellular accumulation of ROS in SH-SY5Y cells (26,28). Although the present study did not investigate whether lycopene could inhibit OGD-induced ER stress, it has previously been reported that lycopene protected cardiomyocytes against the damage induced by hypoxia/reoxygenation via inhibition of ER stress (45,46). Collectively, these findings indicated the inhibitory role of lycopene in OGD-induced oxidative stress and autophagic death may be associated with inhibition of ER stress.

In conclusion, the present study demonstrated that lycopene protected against OGD-induced autophagic death

via inhibition of oxidative stress-dependent activation of the AMPK/mTOR signaling pathway in SH-SY5Y cells. Moreover, it was revealed that the inhibitory effect of lycopene on OGD-induced GSH depletion was associated with the preventive effects of lycopene against OGD-induced depletion of intracellular cysteine and downregulation of xCT. Therefore, lycopene may be considered a potential treatment that could prevent OGD-induced autophagic neuronal death.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL, YZ and YQ conducted the experiments, and acquired, analyzed and interpreted the data. TL drafted the manuscript, figures, and revised the manuscript. TL and YZ confirm the authenticity of all the raw data. YZ and YQ drafted the manuscript and critically revised it for important intellectual content. HL made substantial contributions to the conception and design of the study, conceived and supervised the project, and approved the final version of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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