Regular Article

Protective HSP70 Induction by Z-Ligustilide against Oxygen–Glucose Deprivation Injury via Activation of the MAPK Pathway but Not of HSF1

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Heat-shock protein 70 (HSP70) is known to function as a protective molecular chaperone that is massively induced in response to misfolded proteins following cerebral ischemia. The objective of this study was to characterize HSP70 induction by Z-ligustilide and explore its potential role in protection against cerebral ischemia–reperfusion injury. Our results demonstrated that the intranasal administration of Z-ligustilide reduced infarct volume and improved neurological function in a rat stroke model. Meanwhile, Z-ligustilide enhanced the cell viability of PC12 cells insulted by oxygen–glucose deprivation-reoxygenation (OGD-Reoxy) and decreased apoptotic and necrotic cell death. Importantly, Z-ligustilide induced HSP70 expression both in vitro and in vivo. Although heat-shock factor 1 (HSF1) nuclear translocation was promoted by Z-ligustilide, HSP70-based heat-shock element (HSE)-binding luciferase activity was not activated, and HSP70 expression responsive to Z-ligustilide was not attenuated by HSE decoy oligonucleotides. However, Z-ligustilide significantly activated the phosphorylation of mitogen-activated protein kinases (MAPKs). Further inhibition of MAPK activity by specific inhibitors attenuated HSP70 induction by Z-ligustilide. Meanwhile, downregulation of HSP70 using KMK437, an HSP70 synthesis inhibitor, or small hairpin RNA (shRNA) significantly attenuated the protection of Z-ligustilide against OGD-Reoxy-induced injury. Moreover, the application of specific inhibitors of MAPKs also achieved similar results. Finally, Z-ligustilide alleviated the accumulation of ubiquitinated proteins induced by OGD-Reoxy, which was inhibited by HSP70-shRNA. Taken together, our results demonstrated that Z-ligustilide may induce protective HSP70 expression via the activation of the MAPK pathway, but not canonical HSF1 transcription. HSP70 plays a key role in the protection of Z-ligustilide against OGD-Reoxy-induced injury.

Key words Z-ligustilide; heat-shock protein 70; mitogen-activated protein kinase; oxygen–glucose deprivation-reoxygenation; heat-shock factor 1

The World Health Organization (WHO) Global Infobase reports that stroke has become the second leading cause of mortality in the world, causing around 600000 deaths annually. However, there are currently relatively few treatment options available to minimize damage or death following a stroke. Protein aggregates containing ubiquitinated proteins are commonly present in neurodegenerative disorders and have been considered to cause neuronal degeneration. The previous study also showed that transient cerebral ischemia caused severe protein aggregation in hippocampal CA1 neurons which appeared at 4h and progressively accumulated at 24 and 48h. Therefore, control of protein aggregation may represent an alternative treatment to neurological damage caused by ischemic stroke.

Heat shock proteins (HSPs) are a group of phylogenetically and ubiquitous cytoprotective proteins found in all prokaryotic and eukaryotic cells, many of which are chaperone molecules that facilitate protein folding, trafficking and also prevent their aggregation and degradation. Heat shock protein 70 (HSP70) is a major inducible heat shock protein that basically functions as a molecular chaperone and plays an important role in preventing protein aggregation, degrading unstable and misfolded proteins and transporting proteins between cellular compartments. It’s reported that over-expression of HSP70 via transgenes and viruses or systemic administration of HSP70 fusion proteins that allow it to cross the blood brain barrier protects the brain against ischemia, suggesting that increasing HSP70 level or activity may be a potential therapeutic target for pharmacological intervention of ischemic diseases.

Ligusticum chuanxiong is a commonly used Chinese herbal medicine with its empiric treatment of cardiovascular and cerebrovascular diseases. Z-Ligustilide as the major bioactive phthalide compound of Rhizoma chuanxiong was previously suggested for the prevention and treatment of ischemic stroke. Our and others’ studies showed that Z-ligustilide could protect ischemic injury both in vitro and in vivo via the induction of direct and indirect antioxidant response. However, its effect on the heat shock response, a highly conserved and fundamental cytoprotective mechanism, under ischemic stress remains unexplored. Along this line, we characterized the effect of Z-ligustilide on HSP70 and explored the potential role of HSP70 in the protection of Z-ligustilide against oxygen–glucose deprivation and reoxygenation (OGD-Reoxy) induced injury in this study.
MATERIALS AND METHODS

Materials  Z-Ligustilide was obtained from the Hong Kong Jockey Club Institute of Chinese Medicine (Hong Kong, China) with a purity of >98% and stored in ~80°C before use. HSP70 antibody was purchased from beyotime (Jiangsu, China). The antibody against heat-shock factor 1 (HSF1) was purchased from Enzo Life Sciences (NY, U.S.A.) and lamin b1 was purchased from Santa Cruz Biotechnology (CA, U.S.A.). The antibodies against β-actin and rabbit immunoglobulin G (IgG) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The antibodies against p-p38, p-c-Jun N terminal kinase (JNK) and p-extracellular signal-regulated kinase (ERK) were purchased from Cell Signaling Technology (Boston, MA, U.S.A.). Other chemicals were obtained from Sigma-Aldrich Co., unless indicated otherwise.

Animals and Ischemia–Reperfusion (I/R) Model in Vivo  Sprague-Dawley male (230–260g) rats were offered by the Institute of Laboratory Animals, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. All procedures were approved according to the Animal Care and Use Committee of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital and the Sichuan Province Animal Care Ethics Committee. To test the potential protective effect against the damage induced by I/R, one dose of Z-ligustilide (7.5, 15 or 30 mg/kg) was administered via intranasal route for 3 d. Rats in the sham and vehicle group received saline solution and sliced into 5 coronal sections (2 mm thick). The sections were immediately stained with 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C before use. Cell viability was evaluated by a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, at the end of drug treatment, CCK-8 solution (10µL) was added to each well, followed by incubation for 3h at 37°C. The absorbance at 450nm was determined by a microplate reader (Lambda Bio-20; Beckman). Cell viability was expressed as a percentage of that of the control (untreated) cells.

YO-PRO-1 and Propidium Iodide (PI) Dual Staining  As previously described, cell viability was assessed by YO-PRO-1 (Molecular Probes) and PI (Molecular Probes) dual staining. Briefly, at the end of drug treatment, YO-PRO-1 and PI dyes were added to the culture medium (0.1 µM, and 1.5 µM, respectively) for 30 min. Images were collected with a Zeiss fluorescence microscope (Carl Zeiss, Germany).

Procedure of OGD-Reoxy  To model I/R like conditions in vitro, PC12 cells were exposed to OGD-Reoxy treatment as described previously. In brief, PC12 cells were first incubated in glucose-free DMEM and subsequently transferred into a Tri-Gas incubator (Heal Force, HF100) with 1% O2, 94% N2 and 5% CO2 for 9h at 37°C. Sham OGD cultures were maintained in a normal oxygenated DMEM. Following the OGD treatment, cells were returned to the normoxic incubator with normal culture medium and incubated for another 16h.

Western Blotting Analysis  The cellular proteins were extracted from cerebral cortex or PC12 cells in ice-cold radio immunoprecipitation assay (RIPA) buffer (Cell Signaling Technologies, U.S.A.) supplemented with 1% (v/v) protein inhibitor cocktail and 1 µg/ml phenylmethylsulfonyl fluoride (PMSF) and the nuclear proteins were extracted with nuclear extraction kit (Millipore, U.S.A.). Thirty micrograms of the cellular proteins were resolved by electrophoresis in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membrane. Following 1 h incubation in a fresh TBS buffer containing 0.1% Tween-20 and 5% bovine serum albumin (BSA), the blots were probed with specific primary antibodies. After incubation with the relevant secondary antibodies, the reactive bands were identified using an enhanced chemiluminescence (ECL) detection reagent (GE Healthcare, Sweden). The concentration of the loaded cellular proteins was normalized against the internal control β-actin or lamin b1, and then the value was expressed as each normalized data relative to control.

Luciferase Assay  The luciferase assay was performed as previously described. Briefly, PC12 cells were seeded in 6-well plates and incubated at 37°C in a CO2 incubator for 24h until confluence reached 70–80% and transfected with HSE-luc, a plasmid containing an inducible Rat Hsp70 promoter-driven luciferase reporter gene, by lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. After 24h of growth, cells were treated with either 5 µM Z-ligustilide for up to 4h. As a positive control, cells were heat-shocked at 42°C for 30 min and incubated at 37°C for 3h and 30min. Luciferase assays were performed by using Dual-Luciferase Reporter Assay system (Beyotime, China) according to the manufacturer’s instructions. Firefly luciferase activity was measured by using a luminometer (Lumat LB9502, Berthold, Germany) and the ratio of firefly/luciferase was calculated as a measure of HSE transcriptional activity.
value was normalized to Renilla activity value. Promoter activity was presented as a percentage of change compared with the vehicle-treated control.

**Decoy Design and Treatment** ARE decoy oligonucleotides (ODNs) were used in this study to inhibit HSF1 mediated gene expression according to previous study.21) Upper-strand and reverse-complement phosphorothioated ODNs were commercially synthesized and purified by Sangon Biotech Inc. (Shanghai, China). Double-stranded decoy ODNs were prepared by annealing complimentary single strands in sterile saline. In addition to the HSE decoy ODNs, a scrambled decoy ODNs (mut ODNs) was used as control for specificity.

The following sequences were used in these studies: HSE, 5'-GAT CCT TAT AAG GTC TCC AAA GC-3' and its complement and HSE mut, 5'-TTA GCG CTC ATA GCC AAG TAA TC-3' and its compliment. To determine the transfection rate and cellular localization of HSE decoy ODNs, ODNs were 5'-end-labeled with fluorescein isothiocyanate (FITC). To increase the delivery of ODNs into the cell, lipofectamine 2000 (Invitrogen, U.S.A.) was used in the transfection treatment. The heat-shock element (HSE) decoy and HSE mut ODNs were added to the cells at 100 nM in the presence of lipofectamine 2000. After 24 h of incubation, Z-ligustilide was added directly to the medium as described before.

**Expression Knockdown of HSP70 with KNK437 or HSP70-shRNA** KNK437, N-formyl-3,4-methylenedioxybenzylidene-g-butyrolactam, is an inhibitor of HSP70 synthesis. It was administered 24 h before Z-ligustilide treatment with a concentration of 100 mg/mL.4) Four shRNA sequences targeting HSP70 (Gene ID: 24472) were from the positions (shRNA1, 2317; shRNA2, 2914; shRNA3, 3002; shRNA4, 1552) relative to the start codon and synthesized by GeneChem Co., Ltd. (Shanghai, China). After testing knockdown efficiencies, stem-loop DNA oligonucleotides were synthesized by GeneChem Co., Ltd. (shRNA; sense, 5'-GAT CCG AGG TGC AGG TGA ACT ACA AGG TTC AAG AGA CCT TGT AGT TCA CCT GCA CCT CG-3'; antisense, 5'-AAT TCA AAA AAG AGG TGC AGG TGA ACT ACA AGG TCT CTT GAA CCT TGT AGT TCA CCT GCA CCT CG-3') and cloned into the lentivirus-based RNAi vector pGPU6/GFP/Neo (GeneChem Co., Ltd.). A non-targeting stem-loop DNA was also inserted into pGPU6/GFP/Neo.
vector as a negative control (NC). HSP70-shRNA-lentiviral plasmid and negative control (NC)-shRNA plasmid were respectively packaged with 293T cells using the ViraPower™ Lentiviral Expression Systems (Invitrogen) according to the manufacturer’s protocol. Two days after package, green fluorescent protein (GFP) expression was observed. Lentiviral particles were harvested within 48–72 h post-transfection. PC12 Cells were infected with HSP70-shRNA-lentivirus or NC-shRNA-lentivirus. For each transfection, 1 mL of the lentiviral particles and 1 mL of the complete medium with 6 µg/µL of polybrene were added gently to the cells. After 24 h incubation, the mixture was replaced by 2 mL fresh complete medium for another 24 h. Fluorescent microscope was used to observe the GFP expression. Then, the transfected cells were used for further experiments.

**Statistical Analysis** All data were presented as mean± standard deviation (S.D.) for three independent experiments. Statistical analysis was performed by two-tail Student’s t-test or ANOVA test. A p-value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Z-Ligustilide Protected Brain from Injury Induced by I/R** To address the potential therapeutic role of Z-ligustilide in I/R induced damage, we first examined its effect on rat MCAO model. Rats were pre-treated with Z-ligustilide via intranasal administration for 3 d, once daily before occluding the blood supply. As shown in Figs. 1A and B, infarct volume in 7.5 or 15 mg/kg of Z-ligustilide treated group was significantly smaller than that of vehicle-treated rats at 24 h after ischemia. Besides, our neurological function evaluation showed that pretreatment with 15 mg/kg of Z-ligustilide significantly improved the neurological function at 24 h after ischemia (Fig. 1C). These results demonstrated that Z-ligustilide protected I/R-injured rat brain.

**Protective Effect of Z-Ligustilide against OGD-Reoxy Induced Injury in PC12 Cells** In our previous study, we reported the protective effect of Z-ligustilide against OGD-Reoxy induced injury. In the current study, we confirmed the protection of Z-ligustilide with the concentration of 5 µM, which exhibited the best protective effect in our previous study. As shown in Fig. 1D, Z-ligustilide (5 µM) significantly protected PC12 cells from OGD-Reoxy induced cell death while it also promoted the viability of PC12 cells under normal condition.

Then, we further characterized the type of cell death during this process. As shown in Fig. 1E, control cells displayed negative staining with YO-PRO-1 (which stains apoptotic cells) and PI (which stains dead or dying cells). Exposed to OGD-Reoxy, cells were positively stained with both YO-PRO-1 and PI, indicating that cells underwent both apoptotic death and necrotic death after insulted by OGD-Reoxy. Treatment with Z-ligustilide (5 µM) significantly decreased the positive staining of both YO-PRO-1 and PI, which further confirmed the protective effect of Z-ligustilide.

**Z-Ligustilide Induces HSP70 Expression in Vitro and in Vivo** To determine whether Z-ligustilide induces the expression of HSP70, a protein known to be associated with unfolded and misfolded proteins following cerebral ischemia, we treated PC12 cells with 5 µM of Z-ligustilide for up to 16 h. As a result (Fig. 2A), expression of HSP70 was substantially induced by Z-ligustilide as early as 2 h after treatment. Furthermore, our results showed that Z-ligustilide significantly induced HSP70 expression in a dose-dependent manner within the concentrations of 1.25–5 µM (Fig. 2B).

To determine the potential influence of Z-ligustilide on the HSP70 expression in vivo, we performed Western blotting to
detect HSP70 in the cerebral cortex after I/R. As shown in Fig. 2C, I/R induced a significant increase of HSP70 expression compared with that in the sham group. HSP70 expression further increased when I/R rats were pre-treated with 15 mg/kg of Z-ligustilide.

**Mitogen-Activated Protein Kinases (MAPKs) but Not HSF1 Mediate HSP70 Induction by Z-Ligustilide**

Transcription factor HSF1 is reported to be the key regulator responsible for HSP70 induction.22) To determine the role of HSF1 in the HSP70 induction by Z-ligustilide, we first analyzed the nuclear protein and found that Z-ligustilide promoted the nuclear translocation of HSF1 (Fig. 3A). However, the activity of luciferase reporter carrying HSP70-based HSE did not increase, which demonstrated that Z-ligustilide did not activate HSF1-mediated transcription of HSP70 (Fig. 3B). This result was further confirmed by the fact that HSP70 expression induced by Z-ligustilide didn’t change after inhibition of HSF1 driving downstream genes with HSE decoy ODN (Fig. 3C). Therefore, Z-ligustilide induced HSP70 induction may be not regulated by HSF1 and the upstream mechanisms by which Z-ligustilide activates HSP70 require further investigation.

Recently, a number of studies suggest that HSP70 has been connected to MAPKs signaling pathway.23,24) As HSF1 isn’t the regulator of HSP70 induced by Z-ligustilide, we then asked whether MAPKs pathway plays a possible role in this process. As shown in Figs. 4A–C, upon exposure to Z-ligustilide, phosphorylation of ERK1, 2 peaked at 0.5 h, phosphorylation of p38 peaked at 1 h and phosphorylation of JNK 1, 2 peaked at 1 h as well, suggesting that Z-ligustilide activated MAPKs pathway. To further verify the involvement of MAPKs in the induction of HSP70 by Z-ligustilide, we respectively assessed how Z-ligustilide-induced HSP70 expression was affected by specific kinase inhibitors. As a result, ERK inhibitor PD98059 (Fig. 4D), p38 inhibitor SB203580 (Fig. 4E) and JNK inhibitor SP600125 (Fig. 4F) partially suppressed the expression of HSP70 induced by Z-ligustilide, respectively, suggesting that ERK, JNK and p38 may be involved as upstream targets in the initiation of HSP70 synthesis by Z-ligustilide.

**Z-Ligustilide Ameliorates OGD-Reoxy Induced Injury via HSP70 and MAPKs**

To determine the role of HSP70 in Z-ligustilide mediated protection against OGD-Reoxy induced injury, we further examined whether inhibition of HSP70 synthesis or silencing of HSP70 gene affected the protective effect of HSP70. As shown in Figs. 5A and C, pretreatment with KNK437 and silencing of HSP70 gene by RNAi notably down-regulated the up-regulation of HSP70 expression induced by Z-ligustilide. In *in vitro* OGD-Reoxy model, Z-ligustilide alone significantly protected PC12 cells against OGD-Reoxy induced cell death (*p* < 0.01), whereas this protective effect disappeared in the presence of 100 mg/mL KNK437 (Fig. 5B). Similarly, the HSP70-shRNA also considerably reduced Z-ligustilide mediated protection against OGD-Reoxy (*p* < 0.01) (Fig. 5D). These results suggest that Z-ligustilide reduced OGD-Reoxy induced injury involving HSP70 induction.

Then, we further explored the role of MAPKs activated by Z-ligustilide in its protective effect against OGD-Reoxy induced injury. As showed in Fig. 5E, each of inhibitors (PD98059, SB203580 and SP600125) significantly inhibited Z-ligustilide’s protection of PC12 cells against OGD-Reoxy induced cell death. The results demonstrated that MAPKs pathway may have direct correlation not only with induction of HSP70 but also with Z-ligustilide mediated protection.

**Z-Ligustilide Reduces Ubiquitinated Protein Aggrega-**
tion via HSP70 Induction To study the biological significance of the heat shock response induced by Z-ligustilide, we further determined the effect of Z-ligustilide on the ubiquitinated protein aggregation. As expected, both MG132 (a specific proteasome inhibitor) (Fig. 6A) and OGD-Reoxy (Fig. 6B) caused the accumulation of ubiquitinated proteins. Z-ligustilide significantly reduced the amounts of ubiquitinated proteins induced by either MG132 or OGD-Reoxy ($p<0.01$). Importantly, HSP70-shRNA significantly decreased the alleviating effect of Z-ligustilide on protein aggregation induced by either MG132 or OGD-Reoxy compared with that of NC-shRNA. These findings demonstrated that blocking ubiquitinated protein aggregation may be an important mechanism by which Z-ligustilide protects cells from OGD-Reoxy induced damage via HSP70 induction.

DISCUSSION

Protein aggregation is a biological phenomenon in which abnormal proteins accumulate and clump together either intra- or extra-cellularly. It has been thought to play a pivotal role in the neurodegenerative disease such as Parkinson’s disease and Alzheimer’s disease. Currently, protein aggregation is also regarded as one of the important pathological features of cerebral ischemia and reperfusion (I/R). It has been reported that protein aggregation leads to neuronal death after transient cerebral ischemia. HSPs with their chaperonic function are strongly involved in cellular protection under stressful conditions, which shares the ability to recognize and bind nascent and unfolded proteins, thus preventing aggregation and facilitating proper protein folding. HSP70, a stress inducible chaperone, has been studied for its potential to protect brain from ischemic injury. This protective effect may be mediated by one or more of activities ascribed to HSP70, including refolding denatured proteins and preventing unfolded and damaged proteins from aggregating. Therefore, it has received particular attention as a molecular target for pharmacological intervention of ischemic diseases.

Z-Ligustilide, one of phthalide compounds derived from Rhizoma chuanxiong, exhibited numerous activities in the prevention and treatment of ischemic stroke. However, it has been reported that Z-ligustilide was metabolized into seven metabolites and showed a low bioavailability after oral administration. It is possible that the protective effect of Z-ligustilide via oral administration on I/R injury may be due to the indirect effect exerted by its metabolites. On the other hand, Z-ligustilide could quickly enter the brain after intranasal administration, which implicated that intranasal admin-
administration may be a reasonable way for Z-ligustilide to exert its direct effect on I/R injury. In the current study, our results showed that pre-treatment with Z-ligustilide via intranasal administration significantly protected against rat brain damage induced by transient MCAO in terms of infarct volume and neurobehavioral function, which confirmed the above deduction. Meanwhile, Z-ligustilide also significantly enhanced the cell viability of PC12 cells and decreased the apoptotic and necrotic cell death caused by OGD-Reoxy. In our previous study, Z-ligustilide exhibited antioxidant and cytoprotective effect against OGD-Reoxy injury partially via the activation of Nrf2-HO-1 signaling pathway.10) However, its effect on heat shock response and the role of HSP70 involved in this process remain unexplored. Therefore, the present study was further designed to evaluate the HSP70 induction by Z-ligustilide and the potential role in the protection against I/R injury. Very much as expected, Z-ligustilide significantly induced the expression of HSP70 protein in both in vitro and in vivo. However, our further results demonstrated that HSF1 does not regulate over-expression of HSP70 induced by Z-ligustilide. In support of our results, others’ studies also found that HSF1 rises were not followed by enhanced HSP70 expression.31,32) Recently, cytoprotective functions of HSPs, particularly HSP70, have been connected to MAPKs signaling pathway.33,34) For this reason, we investigated whether there is involvement of MAPKs in the induction of HSP70 by Z-ligustilide. As a result, phosphorylation of the three MAPKs was significantly influenced by Z-ligustilide. Further inhibition of MAPKs activity achieved by respective inhibitors significantly attenuated Z-ligustilide induced HSP70 expression. These results indicate that MAPKs may be involved in the induction of HSP70 by Z-ligustilide as upstream targets.

Fig. 5. Role of HSP70 and MAPKs in the Protection of Z-Ligustilide against OGD-Reoxy Induced Injury

(A) and (C) Western blotting analysis of HSP70 expression in the PC12 cells that treated with KNK437 for 24 h or transfected with HSP70-shRNA-lentivirus or NC-shRNA-lentivirus for 48h, followed by Z-ligustilide (5 µM) treatment for 4 h. The blots were a representative of three independent experiments. (B) and (D) PC12 cells were treated with KNK437 for 24 h or transfected with HSP70-shRNA-lentivirus or NC-shRNA-lentivirus for 48h, followed by Z-ligustilide (5 µM) treatment for 2 h. Then, the cells were subjected to OGD-Reoxy treatment described in Materials and Methods. (E) PC12 cells were treated with 25 µM of PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) or SP600125 (JNK inhibitor) for 1 h, followed by Z-ligustilide (5 µM) treatment for 2 h. Then, the cells were subjected to OGD-Reoxy treatment. The cell viability was determined by CCK-8 assay. Values represent mean±S.D. (n=6). *p<0.05, **p<0.01, ***p<0.001.
showed that inhibition of HSP70 synthesis by KNK437 or silencing of HSP70 gene by HSP70-shRNA significantly attenuated the protection of Z-ligustilide. The results demonstrated that HSP70 protein may play a critical role in this Z-ligustilide mediated protective process. In addition, inhibitors of MAPKs also significantly reduced the protection of Z-ligustilide against OGD-Reoxy induced cell death. These results indicate that transient activation of MAPKs may be a key regulator during the induction of HSP70 and ensuing cell protection by Z-ligustilide.

Recent study demonstrated that overproduction of damaged proteins following focal cerebral ischemia increases protein ubiquitination, resulting in ubiquitinated proteins aggregation.36) Brain ischemia also damages protein degradation pathways, causing the accumulation of ubiquitinated proteins.37) In this study, we also discovered that Z-ligustilide reduced the accumulation of ubiquitinated proteins induced by both MG132 and OGD-Reoxy and provided the evidence that blocking protein aggregation may be an important mechanism for the protection of Z-ligustilide against OGD-Reoxy induced injury. Importantly, HSP70 induction played a key role during this process.

In conclusion, Z-ligustilide via intranasal administration protected brain injury caused by transient MCAO in rats. Additionally, Z-ligustilide protected PC12 cells against OGD-Reoxy induced both apoptotic and necrotic cell death. Transient activation of MAPKs is linked to induction of HSP70 by Z-ligustilide. Moreover, inhibition of HSP70 and MAPKs lead to PC12 cells vulnerable to cell death induced by OGD-Reoxy. Finally, Z-ligustilide reduced the accumulation of ubiquitinated proteins by proteasome inhibitor MG132 and OGD-Reoxy, which was eliminated through knockdown of HSP70. Together, our research suggests that Z-ligustilide may activate endogenous HSP70 against OGD-Reoxy induced damage via non-canonical activation of MAPKs. In our future study, we will further investigate the protective effect of Z-ligustilide on the neurovascular unit against brain injury in the in vivo model and the effect of inhibition of HSP70 up-regulation on this process.

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Conflict of Interest The authors declare no conflict of

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Fig. 6. Z-Ligustilide Alleviates the Accumulation of Ubiquitinated Proteins via HSP70 Induction

(A) PC12 cells were transfected with HSP70 shRNA-lentivirus for 48 h, followed by Z-ligustilide (5 µM) treatment for 2 h. Subsequently, the culture medium was replenished and the cells were incubated in fresh medium overnight. Then, the cells were treated with 500 nM MG132 for 30 min. Cell lysate was used to determine ubiquitinated proteins via Western blotting. (B) PC12 cells were transfected with shRNA-lentivirus for 48 h, followed by Z-ligustilide (5 µM) treatment for 2 h. Subsequently, the cells were subjected to OGD-Reoxy treatment and ubiquitinated proteins were determined as indicated above. Heat shock was carried out at 42°C for 1 h, followed by 37°C overnight. The blots were representative of three independent experiments. *p<0.05, **p<0.01.
interest.

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