Elabela-APLNR Inhibits Cerebral Ischemia/Reperfusion Injury Through AMPK/SIRT3/PGC-1α and Akt/Nrf2 Pathways

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Abstract

Inhibiting mitochondrial dysfunction and oxidative stress has benefits effects for the treatment of cerebral ischemia/reperfusion injury (CI/RI). Elabela/APLENR system had showed to be protective against ischemia/reperfusion induced injury in other tissue. However, whether Elabela had protective effects against CI/RI and its possible mechanisms were largely unknown. This study was designed to evaluate the effects of Elabela 32 (ELA32) against CI/RI.

In vivo, behavioral test, infarct size and brain edema were evaluated on rats middle cerebral artery occlusion/reperfusion (MCAO/R) model after treated by ELA32. *In vitro*, HT22 cells were subjected to glucose deprivation/reperfusion (OGD/R) and treated with ELA32 in presence of SIRT3-siRNA, or AMPK-siRNA, or Nrf2-siRNA. Cell viability, cell apoptosis, ROS and ATP levels, mitochondrial related proteins and oxidative related cytokines were measured by relative methods.

As the results showed, neurological scores, infarct size, brain edema and injury cytokines were improved by ELA32 treatment in rats. Subsequently, we found that ELA32 inhibited cell apoptosis, mitochondrial dysfunction, and oxidative stress in a dose dependent manner in brain and HT22 cell. Western blotting results showed that ELA32 induced the deacetylation and phosphorylation of PGC-1α, the expression of Nrf2, SIRT3 and APLNR, and the phosphorylation of AMPK and Akt. Further, the crosstalk relationship between APLNR, SIRT3, AMPK, PGC-1α, Akt and Nrf2 were verified by the specific targeted siRNA transfection. The same effects on APLNR related pathways were also observed in rats.

In conclusion, ELA32 improved mitochondrial dysfunction and oxidative stress through regulating APLNR mediated AMPK/SIRT3/PGC-1α pathway and Akt/Nrf2 pathway. These results indicated that ELA32/APLNR system plays some role in central nervous system, and more studies should be performed to confirm these effects.

1. Introduction

With the changes of diet and lifestyle, more and more people are diagnosed with the metabolic diseases, like diabetes, hyperlipemia, hypertension, metabolic syndrome, and etc. All these metabolic disorders will induce the changes of microcirculatory flow and blood viscosity in patients, and promote the formation of thrombus in micro- and large-vessels, thus induce ischemia diseases, like myocardial ischemia, cerebral ischemia, renal ischemia[1–5]. Among these, cerebral ischemia has been the third leading cause of death worldwide, and the first leading cause of death and disability in China[6, 7]. Cerebral ischemia is also called as ischemia stroke, which accords for 80% of all strokes[8]. However, the effective treatment protocols are still lack in clinical. To now, the only way of treating ischemia stroke is to quickly restore blood flow using intravenous tissue plasminogen activator (rtPA) and endovascular thrombectomy[9]. However, less than 5% of patients get benefits effects from rtPA because of its narrow therapeutic window (in 4.5 h), and other limited factors[10]. It will result in and/or aggravate secondary brain injury after ischemia injuries when used exceeding the 4.5 h time window, which also called as cerebral

ischemia/reperfusion injury (CI/RI)[11]. In addition, there is no effective treatment that can be used to exhibit long-term recovery improvement. Thus, it is necessary to develop new therapeutic strategies to inhibit CI/RI and improve brain function.

There are various mechanisms involved in CI/RI including excitatory amino acids toxicity, inflammatory response, apoptosis, oxidative stress, mitochondrial dysfunction, and etc. These factors interplay with each others, and aggravate brain injury together [12, 13]. Among these, mitochondrial dysfunction and oxidative stress are found at the beginning of ischemia, and throughout the whole reperfusion process, if mismanaged, they will affect the outcome of treatment [14, 15]. Mitochondrion is the main ATP production organelle in cell. Under normal conditions, mitochondria produce ATP by oxidative phosphorylation through the mitochondrial respiratory chain, and superoxide is the by-product during this process[16, 17]. Superoxide are regulated by the antioxidant enzyme system and converted to water. When ischemia, O_2 is exhausted before glucose, thus anaerobic glycolysis is activated to produce ATP[18]. However, lactic acid and H + are over-produced within the mitochondria, which are converted into H_2O_2 or hydroxyl radical (•OH)[19]. Subsequent reperfusion induces more production of H_2O_2 and •OH which overwhelms endogenous scavenging mechanism[20, 21]. Excessive amounts of ROS induce the oxidative of protein, lipids and DNA in mitochondrial and cell, which aggravate the mitochondrial dysfunction, and also induce cell death by apoptosis or necrosis[22, 23]. According these, increasing antioxidant enzyme levels, decreasing ROS levels and protecting the mitochondrial function are very important for the treatment of CI/RI.

Peptides are small chains of amino acids which have shown therapeutic effects against cardiovascular disease, cerebrovascular disease, metabolic diseases, and other diseases. Among these, Apelin/Apelin receptor (APLNR) system attracts more and more attention. APLNR is an orphaned G-protein-coupled receptor (GPCR), which distributed widely throughout the body, including central nervous system, heart, kidney, and other periphery organs[24, 25]. According to the reports, APLNR has no other subtypes and has two known ligands: Apelin and Elabela[26]. Previous studies had showed that Apelin had protective effects against oxidative induced injuries. In heart, Apelin inhibits mitochondrial oxidative through regulating PI3K/Akt and p44/42 MAPK pathway[27]. In our previous studies, Apelin protected brain cells against I/R-induced ROS mediated inflammation and oxidative stress through activating the AMPK/GSK-3β/Nrf2 pathway[28]. These results indicated that APLNR system has protective effects against oxidative induced injuries. Elabela is newly found endogenous ligand of APJ receptor in 2013, has shown a corresponding role in all aspects of the APLNR system[29–31]. However, its neuroprotective effects against I/R induced oxidative stress and mitochondrial dysfunction in brain is largely unknown to us.

In this study, middle cerebral artery occlusion (MCAO) model *in vivo* and oxygen and glucose deprivation (OGD) *in vitro* model were used to investigate the effects of Elabela on brain cells and further the possible mechanisms were studied.

2. Methods And Materials

2.1 Materials and Reagents

The 32 amino-acid long Elabela 32 peptide (ELA32, QRPVNLTMRRKLRKHNCLQRRCMPLH SRVPFP) with purity more than 98% was synthesized by GenScript (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin-streptomycin solution were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from Biolog industries (BI, Israel). Dihydroethidium (DHE), Dichloro-dihydro-fluorescein diacetate (DCFH-DA) and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma (St. Louis, MO, USA). JC-1 mitochondrial membrane potential detection kit was obtained from Beyotime (Shanghai, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Kaiji Biotechnology (Hangzhou, China). Cell Counting Kit (CCK8) assay kit (ZETA Life Inc., CA, USA). The kits for the detection of ROS, Malondialdehyde (MDA), 8-Oxo-dG, protein carbonyl, Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSH-Px), and Glutathione (GSH) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). MitoSOX Red was obtained from Molecular Probes (Eugene, OR). The primary antibodies for NQO-1, HO-1, P-PGC-1α, PGC-1α, SIRT3, AMPK, P-AMPK, Nrf2, Akt, P-Akt, P-GSK-3β, GSK-3β, APLNR, cleaved-caspase 3, cleaved-caspase 9, Bax, Bcl-2, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were commercial obtained from Chinese suppliers.

2.2 Animal and middle cerebral artery occlusion (MCAO) model

Male Sprague-Dawley rats (6 weeks age, 200-230g) were obtained from the Experimental Animal Center of the Fourth Military Medical University. Rats were kept at the environment with 22-25℃, 45-50% humidity and 12-h light/dark cycle, and free to water and standard cages. The *in vivo* experiments were designed based on the STAIR-criteria and randomization, dose-response assessment, blinding and extensive physiological monitoring were performed strictly. Protocols were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

Rats were divided into sham, model, ELA 32 (30 ng/kg), ELA 32 (60 ng/kg), and ELA 32 (120 ng/kg), randomly, and 15 rats in every group. MCAO model was induced by intraluminal filament method as the direction of previous studies. Briefly, after anesthetized with chloral hydrate (400 mg/kg, ip), the middle cerebral artery was ligated using a 3-0 nylon suture. Laser Doppler flowmetry was used to detect the blood flow, and a blood-flow drop to 80% was considered as successful blockage. After ischemia for 1.5h, the intraluminal suture was withdrawn for reperfusion. ELA 32 was dissolved in saline and intracerebral ventricle injected (3.5 mm depth, ± 1.5 mm mediolateral) 15 min before reperfusion using a stereotaxic frame (Alctt Biotech, Shanghai, China). ELA 32 was given once a day for 3 days.

2.3 Neurological deficit evaluation

Neurological deficit scores were always used to evaluate the neurological function. In this study, a modified scoring method developed from Longa was used. The grade was scored as 0-5: 0 (there was no deficits observed), 1 (rats were failure to extend left forepaw fully), 2 (rats circled to the left), 3 (rats fall to the left), 4 (rats had no spontaneous walking with a depressed level of consciousness) and 5 (dead). The evaluation was performed by two another observers blinding to the study.

2.4 Infarct size measurement

The whole brains were collected and cut into 5 slices with 2 mm thickness. Slices were incubated with 2% TTC solution at 37℃ for 20 min, and fixed in 4% paraformaldehyde. Slices were photographed by a camera and images were analyzed. Infarct size was expressed as percentage of the contralateral hemisphere.

2.5 Cerebral edema measurement

The brain water content was used to evaluate the edema extent. After sacrificed, brains were quickly removed and weighed to get the wet weight (WW). Then, brains were dried at 110℃ for 24h to get the dry weight (DW). The formula for calculating water content (%): (WW - DW)/WW × 100.

2.6 Cell culture

HT22 cell, a mouse hippocampal neuron cell line, was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM medium which contains 10% FBS, and antibiotics in 5% CO_2 at 37℃. The culture medium was changed every other day and subcultured when cell growth to 90% confluence.

2.7 OGD/R model and drug treatments

HT22 cells were seeded into 96 or 6 well plates at appropriate concentration and pretreated with ELA 32 (0.5, 1 and 1.5 μM) for 6h. For OGD, medium was changed with the Earle's balanced salt solution which containing 116 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, l mmol/L NaH_2PO_4, 0.9 mmol/L $CaCl_2$, and 10 mg/L phenol red, and incubated in a hypoxia chamber (Thermo Scientific, USA) pre-gassed with N_2/CO_2 (95%/5%) gas mixture 37℃ for 3 h. After 3 h challenge, culture medium were replaced with the normal medium containing ELA 32, and cultured for another 6h to imitate the reperfusion process. Cells in control group were treated with normal medium in normal condition. Cells in model group were treated with medium without Elabela 32 when reperfusion.

2.8 Cell viability determination

Cells were seeded into 96 well plates and treated as above, and then the supernatant was replaced by normal medium containing Cell Counting Kit (CCK8). After 4h incubation at 37℃, the optical density was assessed at 450nm using a (Bio-Rad Laboratory, Hercules, CA). The results were showed as the fold of control.

2.9 Apoptosis rate determination

After different treatments, the apoptosis rate of HT22 cells were measured using an Annexin V-FITC/PI apoptosis detection kit according to the instruction. The apoptosis was measured by using flow cytometer analysis (BD FACS Aria II).

2.10 ROS measurement

ROS levels in brain tissues were measured by 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and the fluorescence intensity was measured by a spectrofluorometer (Shimadzu Corp., Japan). The results were showed as the fold of sham group. $O_2^{\cdot-}$ levels in mitochondria of HT22 cells were measured by MitoSOX Red. H_2O_2 levels in cells were measured by DCFH-DA. $O_2^{\cdot-}$ levels in cells were measured by DHE. A laser confocal microscope (Nikon, Japan) was used to acquire images.

2.11 Mitochondrial membrane potential (MMP) measurement

The MMP was measured by tetrechloro-tetraethylbenzimidazol carbocyanine iodide (JC-1) as the direction of manufacturer. Briefly, after cells treated with different treatments, JC-1 at a final concentration of 10 μg/ml was added into the cell cultures, and incubated at 37 ℃ for 30min. Cells were washed by PBS and the fluorescence was observed by a laser confocal microscope (Nikon, Japan).

2.12 ATP level measurement

Tissues or cells were lysed sufficiently and centrifuged at 12000g, 4℃ for 10min. The supernatant was mixed with ATP working dilution as 1:1, after incubation, the fluorescence intensity was measured by a spectrofluorometer. The ATP levels were expressed as the fold of control.

2.13 Mitochondrial enzyme activities measurement

Mitochondria were isolated and purified by differential centrifugations. The mitochondria-located enzymes complex I, succinate dehydrogenase (SDH) and complex V were measured by commercial kits as the direction of manufacturer.

2.14 Biochemical analysis

Brain tissues and HT22 cells were lysed by RIPA buffer, and the supernatant was collected for the determination of biochemical values. Levels of MDA, GSH, LDH, CAT, 8-Oxo-dG, protein carbonyl and GSH-Px were measured by commercial kits as the direction of manufacturer.

2.15 siRNA transfection

AMPK, SIRT3, APLNR, Akt, and Nrf2 specific short interfering RNA (siRNA) were designed and chemically synthesized by Shanghai Genechem Company. HT22 cells were transfected by siRNA molecules using the Lipofectamine RNAi MAX reagent as the manufacturer’s direction (Life Technologies, CA, USA). After

transfection for 48h, the transfection efficiency was measured by Western blotting. Tranfected cells were treated with ELA 32 and OGD/R for the further studies.

2.16 Western blotting

After different treatments, brain tissues and HT22 cells were collected, washed by cold PBS and lysed by RIPA buffer with protease inhibitor cocktail. Protein concentrations in samples were measured by BCA protein assay kit. Proteins were separated by 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane. After blocked by 5% nonfat dry milk solution, membranes were incubated at 4 ℃ overnight with the corresponding primary anti-bodies: NQO-1 (1:1000), HO-1 (1:1000), PGC-1α (1:1000), P-PGC-1α (1:1000), SIRT3 (1:8000), AMPK (1:1000), P-AMPK (1:1000), Nrf2 (1:8000), Akt (1:1500), P-Akt (1:1500), P-GSK-3β (1:1000), GSK-3β (1:1000), APLNR (1:800), cleaved-caspase 3 (1:1000), cleaved-caspase 9 (1:1000), Bax (1:1000), Bcl-2 (1:1000), and GAPDH (1:1500). After incubated with secondary antibodies, membranes were visualized by an enhanced chemiluminescent substrate (Thermo, USA). The densities were scanned and quantified using image-analysis systems (Bio-Rad, USA). The results were showed as the fold of control.

2.17 Acetylation assays

PGC-1α acetylation level was measured by immunoprecipitation (IP) followed by Western blotting using antiacetylated lysine antibodies.

2.18 Statistical analysis

Experimental data were collected from triplicate parallel experiments unless otherwise indicated. Results were analyzed by GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) and expressed as mean ± SD. Statistical analyses were performed using One-way ANOVA followed by the Tukey test and $P<0.05$ was considered to be statistically significant.

3. Results

3.1 ELA32 protected brain from MCAO/R induced injuries

The neurological functions were always impaired by I/R. As observed, in sham group, rats had no abnormal condition. After MCAO and reperfusion, rats were listlessness and performed abnormal behaviors. ELA32 treatments improved mental states of rats and reduced the frequency of severe symptoms significantly. The brain functions were evaluated by the neurological deficit scores. As the results showed in Fig.1 A, neurological deficit scores were higher in model group which compared with the sham group, suggesting MCAO/R induced serious neurological deficits in rats. In the ELA32 treatment groups, the neurological deficit scores were lowered in a dose dependent manner, suggesting ELA32 improved neurological deficits in rats.

After neurofunctional assessment, rats were sacrificed. Infarct sizes in brain were measured by TTC straining. As showed in Fig.1 B, the infarct sizes in model group were significantly increased which compared with the sham group($P<0.01$), suggesting MCAO/R induced cerebral infarction in brain, while ELA32 significantly reduced the infarct sizes in a dose dependent manner when compared with model group ($P<0.01$). The cerebral edema extent was measured by the brain water content (Fig.1 C). Compared with the sham group, the water content was significantly increased by MCAO/R ($P<0.01$), suggesting MCAO/R induced cerebral edema. Compared with the model group, the water content was significantly decreased in ELA32 treatments groups, suggesting ELA32 inhibited cerebral edema induced by MCAO/R. Further, the brain injury markers (S-100β and NSE) in serum were measured. As the results showed in Fig.1 D and E, S-100β and NSE levels were both significantly increased in the MCAO/R group ($P<0.01$), however, ELA32 treatment significantly decreased the S-100β and NSE levels in a dose dependent manner. These results suggested that ELA32 protected brain from MCAO/R induced injuries.

3.2 ELA32 improved mitochondrial dysfunction and apoptosis in brain

The apoptosis rate in brain cells were measured by using a TUNEL kit as the direction. As the results showed in Fig.2A, TUNEL positive ratio in model group was significantly higher than that in sham group ($P<0.01$). Compared with model group, TUNEL positive ratio in ELA32 treatments groups were lower than that in model group, and showed a dose dependent manner. Then, we measured the apoptosis related proteins (Bax, Bcl-2, cleaved-caspase 3 and cleaved-caspase 9) in brain tissues (Fig.2B). Bax, cleaved-caspase 3 and cleaved-caspase 9 expression levels were increased and Bcl-2 expression levels were inhibited significantly in the model group when compared with the sham group. In ELA32 treatment groups, Bax, cleaved-caspase 3 and cleaved-caspase 9 expression levels were inhibited and Bcl-2 expression levels were increased significantly ($P<0.01$). These results suggested that ELA32 inhibited cell apoptosis induced by MCAO/R in brain.

Mitochondrial function was evaluated by the ATP levels and mitochondrial respiratory chain related proteins. As the results showed in Fig2.C, ATP levels were decreased by MCAO/R model, and ELA32 increased the ATP contents in a dose dependent manner. We also found that the mitochondrial respiratory chain related proteins including SDH (Fig2.D), complex I (Fig2.E) and complex V (Fig2.F) activities were also decreased in MCAO/R model when compared with sham group. However, ELA32 treatment significantly increased the activities of SDH, complex I and complex V. These results indicated that ELA32 improved mitochondrial function in the brain.

3.3 ELA32 inhibited oxidative stress induced by MCAO/R

To access the effects of ELA32 on the oxidative stress, ROS, MDA, and antioxidant enzymes were measured by kits. As the results showed in Fig.3A and B, the levels of ROS and MDA in brain were increased by MCAO/R, whereas ELA32 decreased ROS and MDA levels significantly ($P<0.01$). The antioxidant enzymes including SOD (Fig.3C), GSH (Fig.3D), GSH-Px (Fig.3E) and CAT (Fig.3F) levels were decreased in the MCAO/R model group. After treated by ELA32, SOD, GSH, CAT and GSH-Px levels were

increased and showed a dose dependent manner. These results showed that ELA32 inhibited oxidative stress induced by MCAO/R.

3.4 ELA32 inhibited cell injuries induced by OGD/R

HT22 cell is a neuron cell line derived from mouse hippocampal and always used as a neuronal cell model in studies of neurotoxicity and ischemic stroke[32, 33]. To study the protective effects of ELA32 on cells directly, HT22 cells were used in *in vitro* studies. As the results showed in Fig.4A, OGD/R decreased cell viability value to 46% of the control cell, suggesting OGD/R induced cell injury in HT22 cell. However, ELA32 at the dose of 0.5, 1.0 and 1.5 μM significantly prevent the decrease of cell viability induced by OGD/R. The cellular morphology showed that large amounts of cells exhibited typical characteristics of apoptosis cell, including cell shrinkage, floating, and cell size reduction and turn round in OGD/R model group, and ELA32 improved these changes (data not shown). Next, the biomarkers of cell injury were measured. As the results showed in Fig.4B and C, NSE and S-100β levels were increased significantly in OGD/R model group, whereas ELA32 decreased NSE and S-100β levels significantly ($P<0.01$). The results of LDH also showed that ELA32 inhibited the increase of LDH induced by OGD/R (Fig.4D, $P<0.01$).

Apoptosis was evaluated by flow cytometry and western blotting. As the results showed in Fig.4E, OGD/R induced 45% cells apoptosis in model group, and the apoptosis rates were 35%, 27% and 19% in 0.5, 1.0 and 1.5 μM ELA32 group, respectively. The protein expression levels of Bax, and cleaved-caspase 3 were increased and Bcl-2 was decreased in the OGD/R model group which compared with the control group (Fig. 4F, $P<0.01$). In the ELA32 treatment groups, Bax and cleaved-caspase 3 expression levels were decreased and Bcl-2 expression levels were increased significantly compared with the model group (Fig. 4G, $P<0.01$). These results suggested that ELA32 inhibited cell injury induced by OGD/R.

3.5 ELA32 inhibited oxidative stress and mitochondrial dysfunction induced by OGD/R

The oxidative and mitochondrial function related indicators were measured in the further studies. To observe the ROS levels in cells directly, cells were stained by DCFH-DA and MitoSOX Red. As the results showed, the fluorescence intensity of DCFH-DA and MitoSOX Red in model group were significantly increased which compared with control group (Fig. 5A and B). The oxidation product of lipid, MDA (Fig. 5C), the oxidation product of protein, protein carbonylation (Fig. 5D), and the oxidation product of nucleic acid, 8-Oxo-dG (Fig. 5E) were increased in the OGD/R treated cells ($P<0.01$). However, the fluorescence intensity of DCFH-DA and MitoSOX Red were decreased and the levels of MDA, protein carbonyl and 8-Oxo-dG were also decreased in the ELA32 treatment groups and showed a dose dependent manner ($P<0.01$). Anti-oxidant proteins SOD (Fig.5F), GSH (Fig.5G) and CAT (Fig.5H) levels were also measured by kits. OGD/R induced the decrease of SOD and CAT levels in cells and ELA32 reversed these effects ($P<0.01$). These results suggested that ELA32 inhibited oxidative stress induced by OGD/R in HT22 cells.

To evaluate the mitochondrial function, mitochondria related factors were measured. MMP was measured by JC-1 staining which accumulated as a J-aggregative form in the mitochondria and showed the red fluorescence. In the OGD/R treated cells, the red fluorescence reduction and green fluorescence

increasing were observed (Fig. 6A). By contrast, ELA32 treatment significantly inhibited OGD/R induced MMP dysfunction. Cytochrome C always used to evaluate the mitochondrial function. As the results showed in Fig. 6B, OGD/R induced cytochrome C expression in cells, and ELA32 could inhibit the protein expression of cytochrome C in a dose dependent manner. OGD/R induced the decrease of SOD2, UCP2, complex I and complex V protein expression levels in cells, whereas ELA32 increased the expression levels of these proteins (Fig. 6C). The production of ATP also decreased by OGD/R treatment, and ELA32 restored the ATP levels to meet the needs of the cells (Fig. 6D). And also the activity of SDH, complex I and complex V were increased by ELA32 treatments (Fig. 6E-G).

3.6 ELA32 regulated SIRT3/PGC-1α through AMPK

To explore the possible mechanism of ELA32 on mitochondrial functions improvement, AMPK/SIRT3/PGC-1α pathway was studied. As the western blotting results showed in Fig.7A, OGD/R induced dephosphorylation of PGC-1α, almost 80% less than that in the control group, the main two downstream NRF1 and TFAM also decreased significantly after OGD/R treatment (P<0.01). However, ELA32 treatment induced the phosphorylation of PGC-1α and also the protein expression of NRF1 and TFAM. OGD/R also induced acetylation of PGC-1α, which caused inactivation of PGC-1α, in contrast, ELA32 inhibited acetylation of PGC-1α in a dose dependent manner (Fig.7B). After OGD/R treatment, the phosphorylation levels of AMPK and the expression levels of SIRT3 were decreased significantly, which compared with that in control group (P<0.01). Compared with OGD/R model group, ELA32 treatment increased AMPK phosphorylation and SIRT3 expression levels in HT22 cells (Fig.7C).

To confirm whether the protective effect of ELA32 was through regulating AMPK/SIRT3/PGC-1α pathway, siRNA target AMPK and SIRT3 plasmids were used in the further studies. As the results showed in Fig.7D, siAMPK induced the decreased SIRT3 expression and phosphorylation levels of PGC-1α, and also the expression levels of NRF1 and TFAM, which compared with the scrb group (P<0.01). The effects of ELA32 on ATP levels (Fig.7E), mitochondrial membrane potential (Fig.7F) and cell viability (Fig.7G) were abolished by siAMPK treatment, indicating that the protective effects of ELA32 on mitochondrial functions were destroyed by siAMPK treatment. From the results shown in Fig.8A, we found that siSIRT3 abolished the deacetylation effects of ELA32 on PGC-1α (Fig.8A), and also the protein expression levels of NRF1 and TFAM (Fig.8B). The effects of ELA32 on ATP production were also abolished by siSIRT3 treatment (Fig.8C). These results indicated that the protective effect of ELA32 was through regulating AMPK/SIRT3/PGC-1α pathway.

3.7 ELA32 regulated Nrf2/ARE through Akt

To study the possible anti-oxidant mechanisms of ELA32, Akt/Nrf2 pathway was examined. From the results of fluorescence reporter gene analysis (Fig.9A), we found that ELA32 significantly increased the luciferase activity of ARE. In Fig.9B, the results showed that ELA32 induced the protein expression levels of Nrf2, and it's downstream, NQO-1 and HO-1 in cytoplasm. We also found the expression levels of Nrf2 were increased in the nuclear, indicating ELA32 treatment induced Nrf2 expression and transfer into the nuclear (Fig.9C). To confirm the role of Nrf2, Nrf2-specific siRNA was used. After siRNA transfection,

HT22 cells were treated with ELA32 (1.5 μM) and OGD/R. As the results showed in Fig.9D, compared with the scrb group, the protein expression levels of Nrf2, NQO-1 and HO-1 were decreased after siNrf2 transfection. Effects of ELA32 on ROS levels (Fig.9E), SOD activity (Fig.9F) and cell viability (Fig.9G) were also abolished by siNrf2 transfection. These results indicated that Nrf2 played an important role in the protective of ELA32.

To study the upstream of Nrf2/ARE activation, Akt was studied in the further studies. Compared with the control group, OGD/R decreased the phosphorylation level of Akt (Fig. 9H). However, ELA32 treatments induced the increase of Akt phosphorylation in a dose dependent manner. Next, Akt-specific siRNA was used to verify whether ELA32 induced Nrf2/ARE activation through Akt. As the results showed in Fig.9I, compared with scrb group, the increase expression of Nrf2 induced by ELA32 was inhibited by siAkt transfection. And also effects of ELA32 on ROS (Fig.9J) and SOD (Fig.9K) were abolished by siAkt transfection. These resulted showed that ELA32 induced the expression of Nrf2 through Akt.

3.8 ELA32 regulated AMPK and Akt through APLNR

Firstly, the protein expression level of APLNR was measured. As the results showed in Fig.10A, ELA32 treatment significantly increased the protein expression levels of APLNR which compared with the control group (P<0.01). To confirm the role of APLNR, APLNR-specific siRNA was used. Western blotting results showed that siAPLNR significantly deceased the phosphorylation of AMPK, Akt, PGC-1α, and the expression of SIRT3 and Nrf2 which induced by ELA32 treatment (Fig.10B). In siAPLNR transfected cells, the effects of ELA32 on mitochondrial membrane potential (Fig.10C), ROS levels (Fig.10D), ATP levels (Fig.10E) and cell viability (Fig.10F) were found to be inhibited when compared with scrb group. These results showed that ELA32 regulated AMPK and Akt pathway through APLNR.

3.9 ELA32 regulated APLNR related pathways in rats

To determine whether the APLNR/AMPK/Akt was involved *in vivo*, brain tissues were collected and protein expression levels were measured by Western bloting. As the results showed in Fig. 11, MCAO/R induced the decrease of the protein expression levels of APLNR, SIRT3 and Nrf2, and the phosphorylation level of AMPK, Akt and PGC-1α. However, in the ELA32 treatment groups, the protein expression levels of APLNR, SIRT3 and Nrf2, and the phosphorylation level of AMPK, Akt and PGC-1α were increased significantly (P<0.01). These results indicated that APLNR/AMPK/Akt pathway may be involved in the protective effects of ELA32 *in vivo*.

4. Discussion

Stroke has been one of the widespread risk triggers of morbidity worldwide and a important cause for permanent adult disablement in human community[34]. According to the statistical findings of the Global Burden of Disease study, number of stroke cases in low- and middle-income countries accord for 70% of all strokes in word[35]. Long-terms medical treatments and physical disability bring medical and financial burdens to the social and family. Two main types of stroke are diagnosed in clinical, ischemic stroke and

hemorrhagic stroke, among these, ischemic accounts for the majority[36]. For the treatment of ischemic stroke, there are two main therapeutic strategies: reperfusion and neuroprotection are commonly used in clinical. For reperfusion, thrombolytic, antithrombotic and anti-aggregation drugs are used. However, the narrow therapeutic time window and the associated bleeding risk limit the clinical use severely[37]. For neuroprotection, neuroprotective agents are used to prevent cell death through regulating cellular pathways relating cell injuries induced by stroke after reperfusion. Ischemic penumbra can be restored by using neuroprotective agents, thus save the tissue and cells in penumbra and limit the negative results of stroke[38]. However, there is no such neuroprotective agent with proven efficiency and without adverse side effects used in clinical. Therefore, it is necessary to develop new agents to treat or prevent I/R induced brain injuries.

Elabela, also called as Apela or Toddler, is encoded by a gene NM_001297550 located at a region of the human genome and annotated as non-coding RNA which is highly conserved in vertebrates[39]. Elabela is a natural peptide of Apelin receptor (APLNR, also known as APJ), and has many functional similarities to Apelin, another natural peptide of APLNR. Elabela was found in 2013, and Apelin was found in 1998, so many effects of APLNR were explored by Apelin. To reveal the biological functions of Elabela, more and more works were initiated, and the knowledge about Elabela is slowly enriched. It had proven that Elabela had the same effects with Apelin on many sides, such as angiogenesis, fluid homeostasis, maintaining cell growth and selfrenewal, and so on. In kidney, Elabela inhibited acute kidney injury through inhibiting apoptosis, inflammation, fibrosis and autophagy *in vitro* and *in vivo*[40]. Elabela was also found that it acts as a long non-coding RNA and binds with hnRNPL to decrease the interaction between hnRNPL and p53[41]. In CNS, Elabela regulates food and water intake through activating arginine vasopressin (AVP) and corticotropinreleasing hormone (CRH) neurons in the paraventricular nucleus (PVN)[42], indicating that Elabela may interacts with APLNR in CNS. However, the biological properties and functions of Elabela on stroke were largely unknown to us. This study was aimed to evaluate the effects of Elabela on stroke and further illustrate the possible mechanism.

In this study, MCAO/R induced rat I/R model was used *in vivo*. MCAO/R induced serious neurological deficits, increased infarct sizes, and caused cerebral edema in rats, and the brain injury markers (S-100β and NSE) in serum were also increased significantly. Treatments with ELA32 improved neurological functions, decreased infarct sizes, cerebral edema and the levels of brain injury markers, indicating ELA32 had protective effects against MCAO/R induced injuries. In *in vitro*, HT22 cell, a mouse hippocampal neuron cell line, was used and OGD/R model was taken to imitate I/R model. The results also showed that ELA32 increased cell viability, decreased LDH, NSE and S-100β levels which induced by OGD/R. Apoptosis plays as an important role in brain injuries or dysfunction induced by I/R[43]. In the study, we also found that MCAO/R or OGD/R induced apoptosis in brain tissues or HT22 cells which detected by TUNEL and flow cytometry. Western blotting results showed the mitochondrial apoptotic pathway related proapoptosis proteins (Bax, cleaved-caspase 3 and cleaved-caspase 9) were increased by MCAO/R or OGD/R. ELA32 decreased apoptosis ratio and the expression levels of proapoptosis proteins, indicating ELA32 treatment had inhibition effects on cell apoptosis.

In human tissues, brain is not the biggest tissue (only 2% of the body weight), but it consumes 20% of the energy in the body[44]. To produce enough energy, brain consumes a large quantity of oxygen and needs lots of mitochondria, which makes it vulnerable to oxidative stress[45]. Oxidative stress was defined as the balance of pro-oxidant and antioxidant is disturbed by some factors, like I/R[46]. In mitochondria, ATP is produced by mitochondrial electron transport, and ROS is by-produced during this process and cleaned by antioxidant proteins (SOD, CAT, GSH, and so on)[47]. When ischemia, anaerobic glycolysis is initiated to produce ATP, meanwhile, large amounts of ROS are by-produced, which induce endogenous antioxidant capacity overwhelmed, thus the levels of superoxide and hydrogen peroxide are uncontrolled[18, 48]. Mitochondrial DNA and protein will be oxidized by superoxide which aggravate the mitochondrial dysfunction and induce mitochondrial related cell apoptosis during CI/R[49]. It thus appears that inhibiting oxidative stress and improving mitochondrial dysfunction will prevent the injury induced by CI/R.

In present study, ROS and MDA levels were increased in MCAO/R model, and the oxidation product of nucleic acid, 8-Oxo-dG and the oxidation product of protein, protein carbonylation, were increased after OGD/R treatment in HT22 cells. The antioxidant proteins (SOD, GSH and CAT) were both inhibited by MCAO/R or OGD/R, indicating oxidative stress was induced in brain and HT22 cell. ELA32 treatment inhibited ROS levels and oxidant products of lipid, protein and DNA, increased the levels of antioxidant proteins. These results showed that ELA32 inhibited oxidative stress in brain tissue and cells. Following, the mitochondrial functions were measured by JC-1 and other related factors. OGD/R induced the decease of mitochondrial membrane potential, the release of cytochrome C, the reduction of ATP production, and the inhibition of SOD2, UCP2, SDH, complex I and complex V protein expression, indicating OGD/R induced mitochondrial dysfunction in HT22 cells. ELA32 treatment reversed these changes, improved the mitochondrial function. Above these, the benefits effects ELA32 might through improving mitochondrial function and inhibiting oxidative stress.

Next, the possible mechanisms of ELA32 in inhibiting oxidative stress and improving mitochondrial function were explored. In the cells or tissues, PGC-1 family including PGC-1α and other members are major stimulators of mitochondrial biogenesis and respiration in response to changes[50]. As known, PGC-1α has been considered as the important regulator of cell metabolism and oxidative stress, except mitochondrial biogenesis[51]. PGC-1α regulates the expression of SOD2 which is a major antioxidant enzyme in mitochondria[52]. Meanwhile, PGC-1α inhibits the formation of ROS through regulating the protein expression of UCP2 and UCP3[53]. In brain neurons, higher levels of PGC-1α protect cells from oxidative stress induced cell death, and knockdown of PGC-1α caused the cells sensitivity to oxidative stress and induced cell apoptosis [51, 54]. In this study, the phosphorylation level of PGC-1α was decreased and the acetylation level of PGC-1α was increased, while ELA32 treatment increased the phosphorylation level of PGC-1α, and the expression levels of its downstream, NRF1 and TFAM, besides, ELA32 inhibited acetylation of PGC-1α, indicating ELA32 induced PGC-1α activation in HT22 cells.

Then, the upstream of PGC-1α was explored. Sirtuin 3 (Sirt3) is a NAD^+ dependent deacetylase, which locates in the mitochondria and plays an essential role in regulating mitochondrial homeostasis and

biology, including ATP generation and ROS detoxification[55]. Previous study showed that SIRT3 promoted mitochondrial biogenesis through PGC-1α deacetylation and increased rates of transcription[56]. In OGD/R induced model, the protein expression levels of SIRT3 were decreased and ELA32 reversed this trend. In siRNA-SIRT3 transfected cells, ELA32 lost the deacetylation effects on PGC-1α, and the transcription effects on NRF1 and TFAM. These results indicated that the PGC-1α deacetylation effect of ELA32 was through regulating SIRT3 expression. AMP-activated protein kinase (AMPK), a serine/threonine kinase, has been represented as the “energy sensor” that senses energy status[57]. The nutrient stresses (including ischemia and hypoxia) deplete ATP levels to alter the AMP-ATP ratio, thus activate AMPK[58]. The activation of AMPK regulates several targets to produce ATP and battle against cell damages induced by stresses. In previous study, it has been shown that AMPK regulated stress responses and survival of cells by modulating the SIRT3 expression[59]. In spinal cord neurons, the activation of AMPK protected cells from ischemia injury through regulating PGC-1α pathway [60]. In present study, the phosphorylation level of AMPK was decreased by OGD/R stimulation, and ELA32 restored the AMPK phosphorylation level. Further to confirm the role of AMPK in regulating PGC-1α and SIRT3, siRNA targeted AMPK was used. As expected, siAMPK decreased the effects of ELA32 on regulating PGC-1α and SIRT3, and also the protective effects of ELA32 were abolished by siAMPK. These results indicated that ELA32 regulated PGC-1α activation through modulating the AMPK/SIRT3 pathway.

As reported, the antioxidant proteins are not limited to those proteins in mitochondria, SOD, CAT, and GSH-Px are located totally or partly in the cytoplasm[51]. Large amounts of these antioxidant proteins are regulated by nuclear factor E2-related factor 2 (Nrf2) which binds with antioxidant responsive element (ARE) in nuclear response to stress stimulation [61, 62]. Under normal condition, Nrf2 binds with Kelch-like ECH-associated protein 1 (Keap1) and be degraded by ubiquitin[63]. When cells subjected to stresses, like oxidative stress, Nrf2 is released from Keap1 and translocates into nucleaus, binds to AREs, and induces proteins expression involving heme oxygenase 1 (HO-1), CAT, SOD, GSH-Px, NQO1, γ-glutamyl cysteine sythetase (γ-GCS), and other kinds of antioxidant protein[64–66]. According to the results of ELA32 on antioxidant proteins (CAT, GSH-Px, SOD) expression in cytoplasm, we assumed that ELA32 had some effects on Nrf2 pathway. Interesting, the results showed that ELA32 induced Nrf2 expression and nuclear translocation, and the increase of ARE transcriptional activity in HT22 cells, indicating ELA32 activated Nrf2/ARE axis in HT22. Next, siRNA-target Nrf2 was used to confirm the effect of Nrf2. Compared with scrb transfection, siNrf2 transfection decreased the effects of ELA32 on antioxidant protein activity and abolished its protective effects, suggesting Nrf2 was important for the protective effect of ELA32. Lots of literatures had showed that PI3K/Akt plays an important role in regulating Nrf2-ARE pathway activation [67–69]. In this study, we found that ELA32 induced the phosphorylation of Akt in accordance with the increase of Nrf2 activation. Further, siAkt transfection not only decreased the effects of ELA32 on Nrf2 expression, also abolished the anti-oxidative effects of ELA32. These results suggested that Akt/Nrf2 was essential for the anti-oxidative effects of ELA32.

The activity of Elabela and Apelin are both performed through APLNR, a classical G protein-coupled receptor (GPCR) and the downstream signaling involving AMPK and Akt[70, 71]. As reported, the

Elabela/APLNR axis plays important roles in the circulatory system and embryonic development, but it also has important effects in CNS [72]. To verify whether ELA32 regulated AMPK and Akt through APLNR, siRNA target APLNR was performed. As expected, siAPLNR abolished the effects of ELA32 in regulating AMPK and Akt and their downstream proteins. Also, siAPLNR inhibited the effects of ELA32 on cell viability, mitochondrial functions and ROS levels. These results indicated that the regulation effects of ELA32 on AMPK and Akt were through APLNR.

5. Conclusions

In summary, ELA32 protected brain cells from cerebral ischemia/reperfusion though improving mitochondrial function and inhibiting oxidative stress. The possible mechanisms might through eliciting APLNR mediated AMPK/SIRT3/PGC-1α pathway and Akt/Nrf2 pathway. These results provided evidences for the protective effects of ELA32 in inhibiting cerebral ischemia/reperfusion induced cell injuries.

6. Declarations

Ethics approval and consent to participate

All of the protocols in this study were approved by the Ethics Committee for Animal Experimentation and performed according to the Guidelines for Animal Experimentation of the Fourth Military Medical University and the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All animal experiments were approved by Fourth Military Medical University Animal Care and Use Committee and were designed to minimize suffering and the number of animals used

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that they have no conflict of interest

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Authors' contributions

JWW and JLD designed the experiments, analyzed the results, and drafted the manuscript. SJH and HXC carried out the behavioral measure, ELASA, and immunofluorescence. DMH and FM carried out the western blotting analysis. SJH helped carry out the cell cultures. All authors read and approved the final manuscript.

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Figures

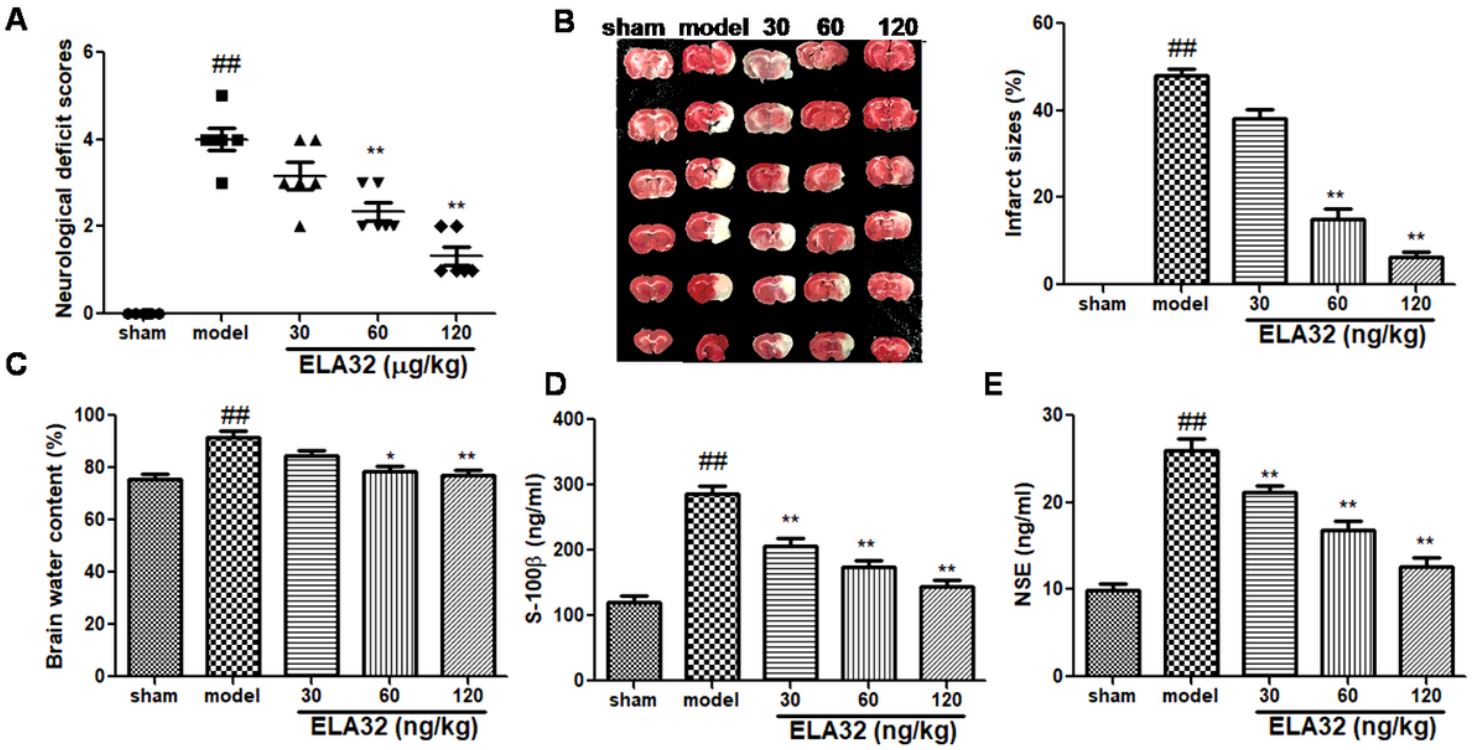

Figure 1

ELA32 inhibited injuries induced by MCAO/R. Elabela 32 was intracerebral ventricle injected 15 min before reperfusion and given once a day for 3 days after MCAO. Brains and serum were collected for different experiments. A. Neurological deficit score was evaluated by the modified Longa's method. B. Infarct sizes were analyzed by TTC staining. C. Brain water contents were measured to evaluate the

degree of cerebral edema. D. S-100β levels in the serum were measured by ELISA kits. E. NSE levels in the serum were measured by ELISA kits. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the sham group; *P < 0.05, **P < 0.01 vs. the model group.

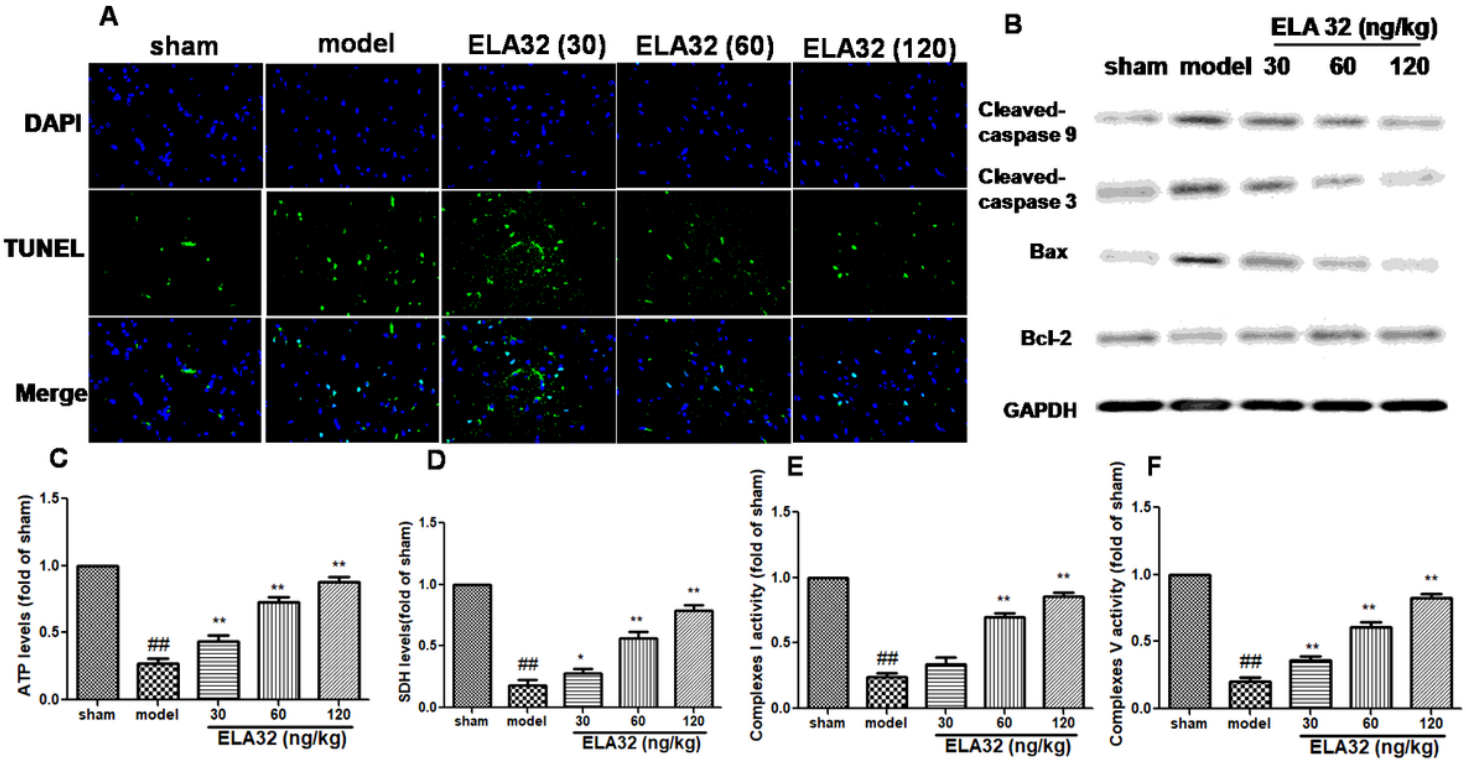

Figure 2

Effects of ELA32 on the apoptosis and mitochondrial function. A. Apoptosis in brain was measured by TUNEL staining (×400). B. Apoptosis related proteins were measured by western blotting. C. ATP levels in brain. D. SDH levels. E. Complexes I levels. F. Complexes V levels. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the sham group; *P < 0.05, **P < 0.01 vs. the model group.

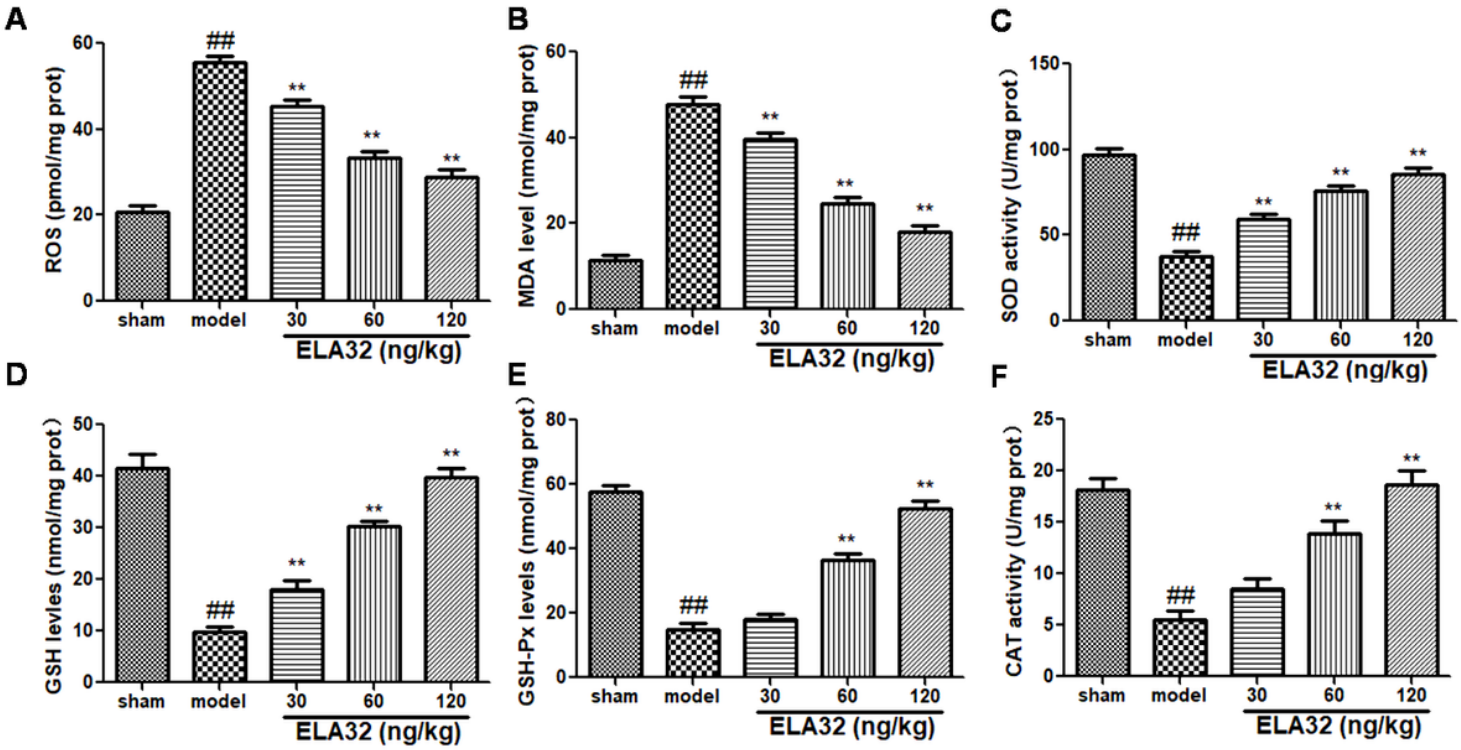

Figure 3

Effects of ELA32 on the levels of oxidative related cytokines. A. ROS levels were measured by a ROS determination kit, B. MDA levels in brain; C. SOD activity; D. GSH levels; E. GSH-Px levels; F.CAT activity in brain. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the sham group; **P < 0.01 vs. the model group.

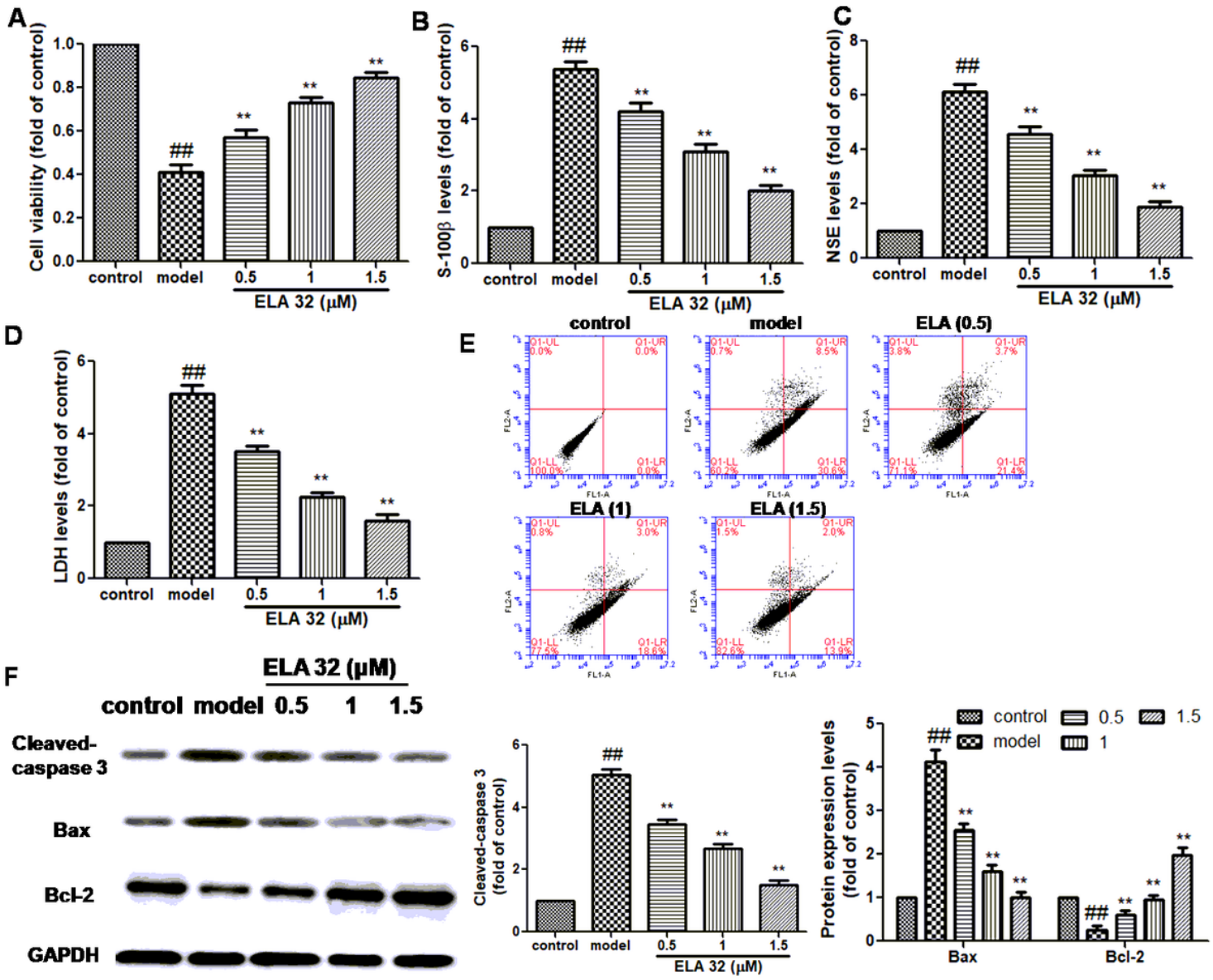

Figure 4

Effects of ELA32 on the cell injuries induced by OGD/R. A. Cell viability. B. S-100β levels in culture medium. C. NSE levels in culture medium. D. LDH levels in culture medium. E. Flow cytometry analysis after Annexin V-FITC and PI staining. F. Apoptosis related proteins expression levels. Representative blot was showed from four independent experiments. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group.

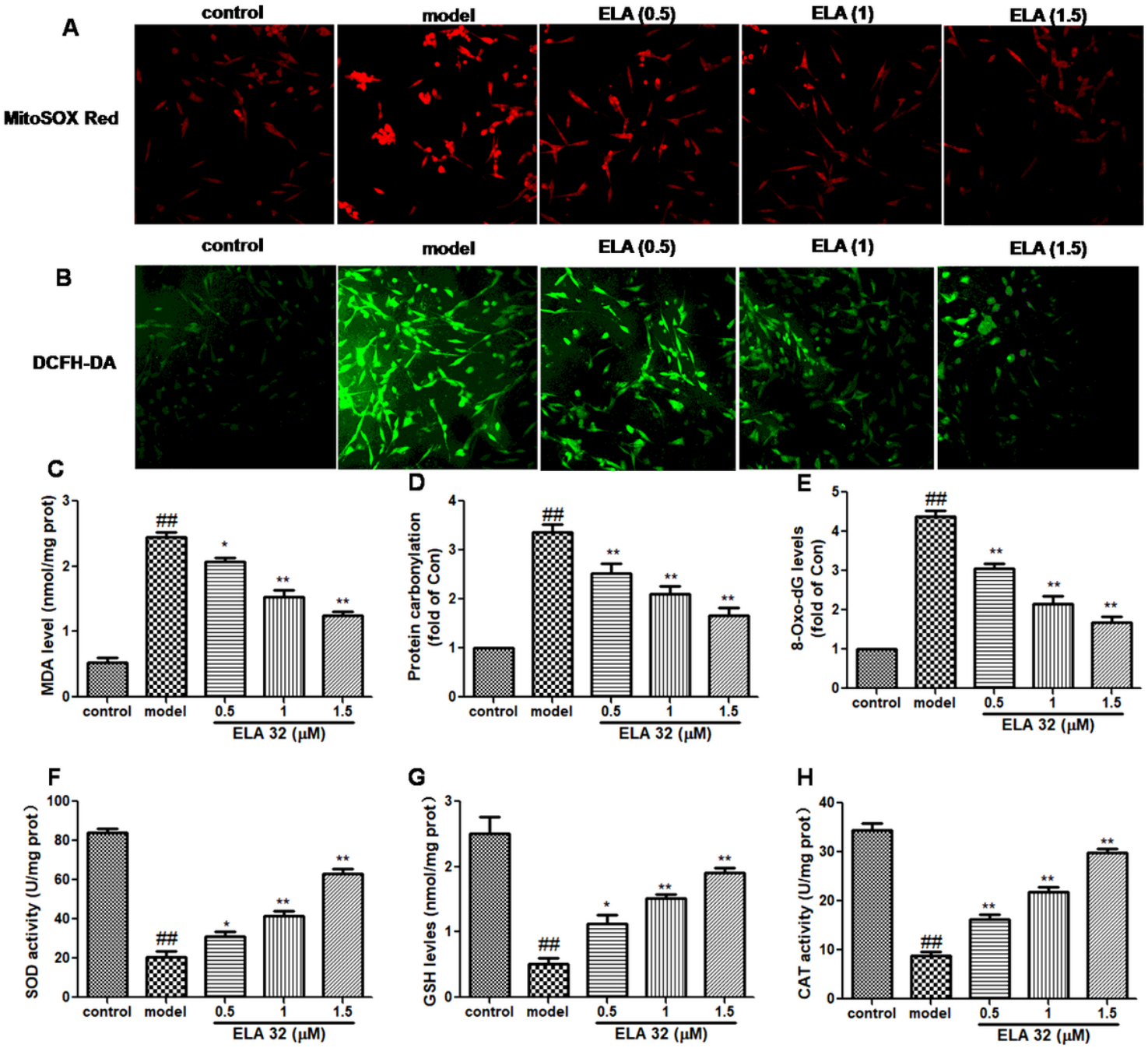

Figure 5

Effects of ELA32 on the oxidative stress induced by OGD/R in HT22 cells. A. MitoSOX Red staining for the detection of mitochondrial oxygen free radical. B. DCFH-DA staining for the detection of ROS in HT22 cells. C. MDA levels. D. Protein carbonylation levels. E. 8-Oxo-dG levels. F. SOD activity. G. GSH levels. H. CAT activity. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; *P < 0.05, **P < 0.01 vs. the model group.

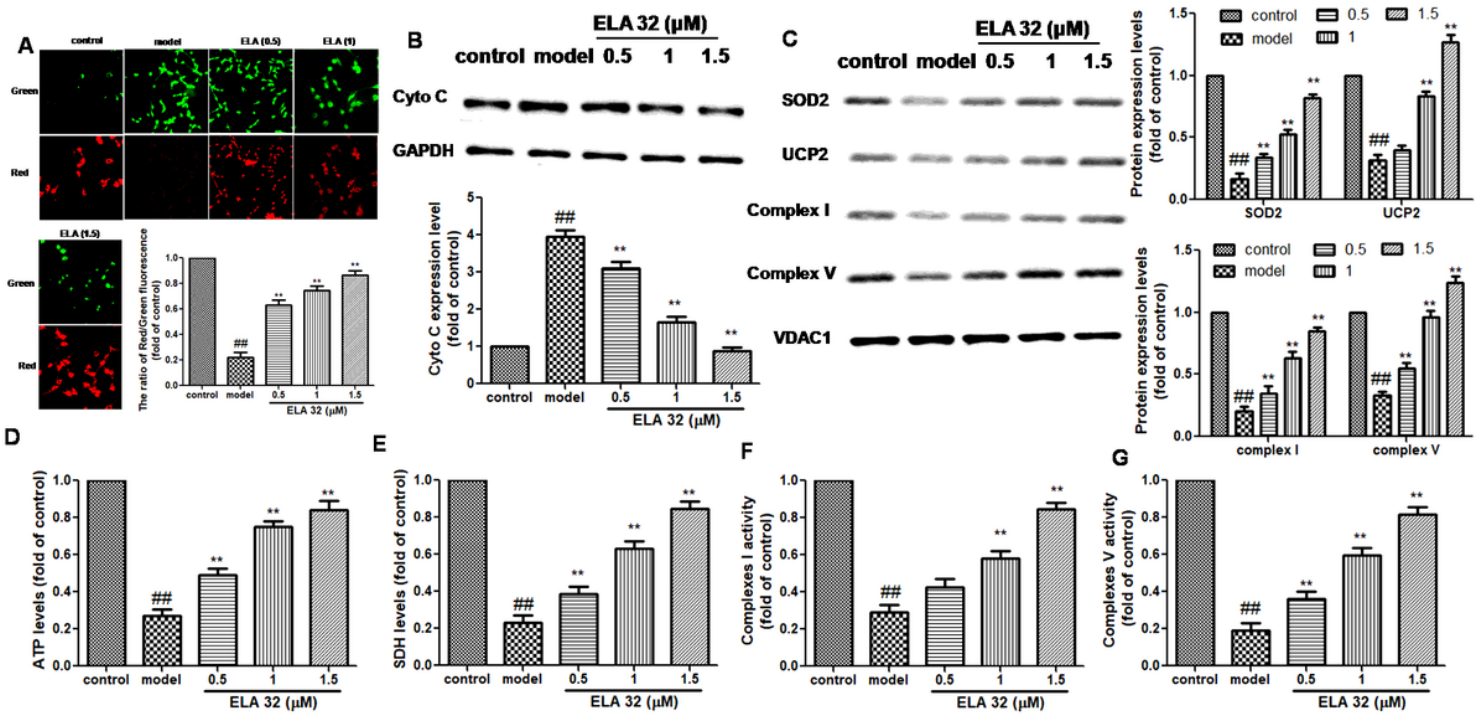

Figure 6

Effects of ELA32 on the mitochondrial functions. A. JC-1 at a final concentration of 10 μg/ml was used to stain the cells. Representative images were showed from five independent experiments (×200 magnification). B. Western blotting results of Cyto C in HT22 cells. C. Western blotting results of SOD2, UCP2, Complex I and Complex V in mitochondria. D. ATP levels. E. SDH levels. F. Complex I activity. G. Complex V activity. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group.

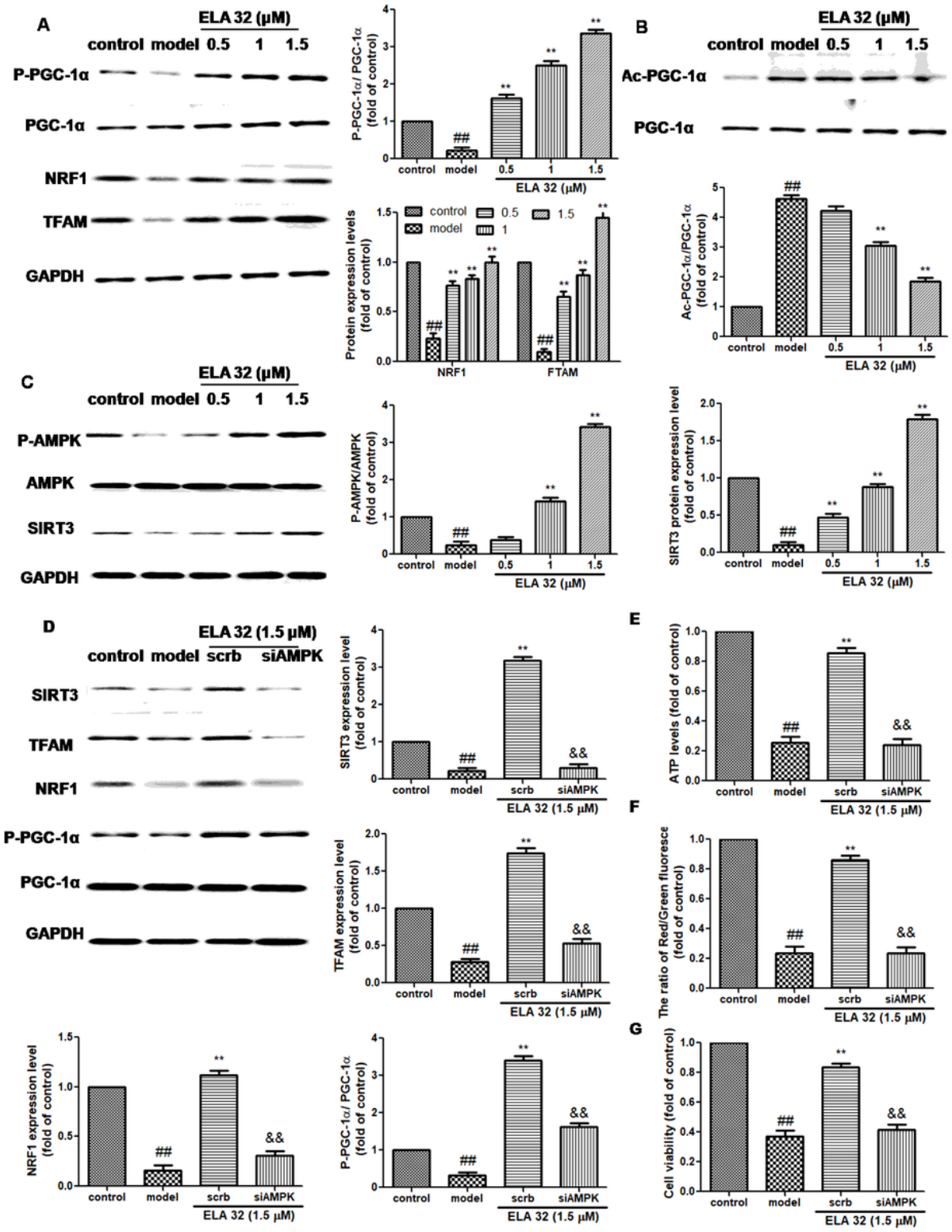

Figure 7

Effects of ELA32 were through regulating AMPK/SIRT3/PGC-1α pathway. A. P-PGC-1α, PGC-1α, NRF1 and TFAM protein expression levels were measured by western blotting with relative antibodies. B. Ac-PGC-1α was immunoprecipitated using PGC-1α and antiacetylated lysine antibodies. C. Effects of ELA32 on the expression of P-AMPK, AMPK, and SIRT3. D. HT22 cells were transfected with AMPK siRNA for 48 h, and then treated with ELA32 (1.5 μM) and OGD/R. SIRT3, P-PGC-1α, PGC-1α, NRF1 and TFAM protein

expression levels were measured by western blotting with relative antibodies. ATP (E), mitochondrial membrane potential (F) and cell viability (G) were measured by relative kits. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group, &&P < 0.01 vs. the scrb group.

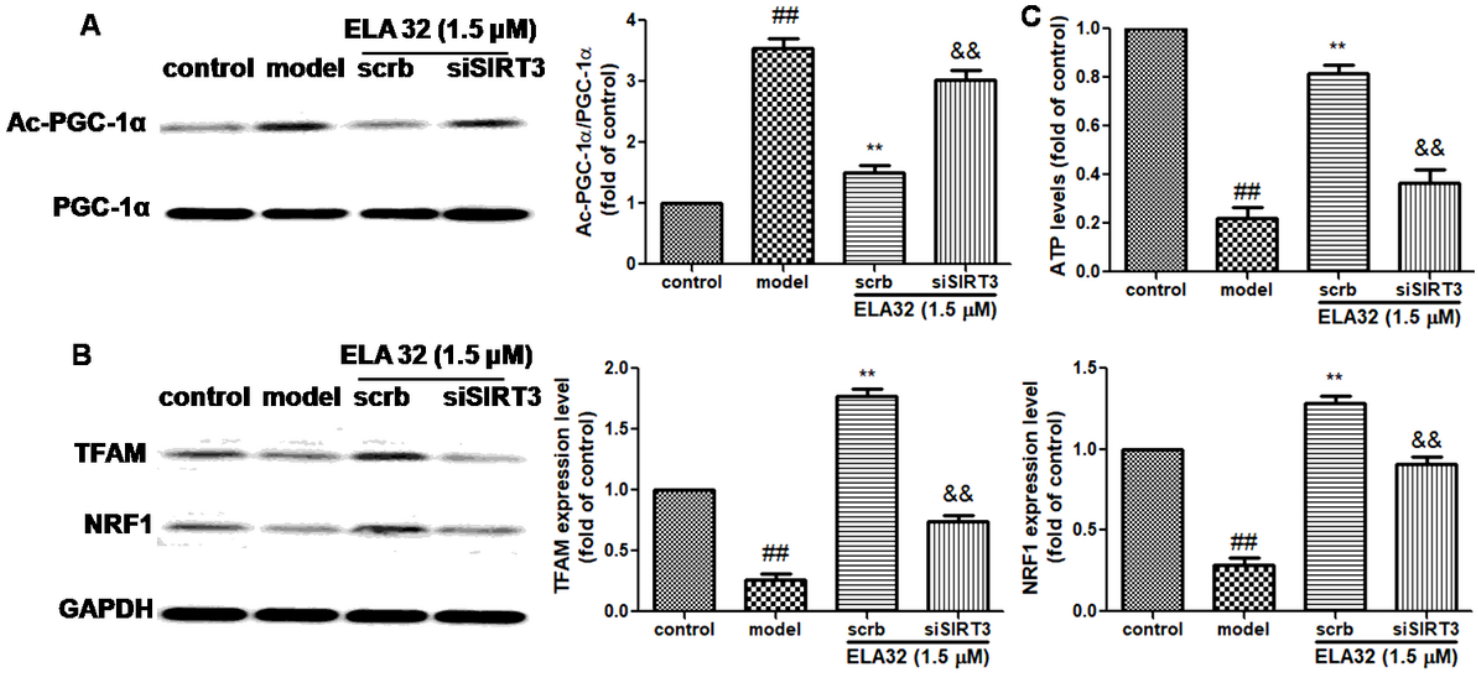

Figure 8

Effects of ELA32 were through regulating SIRT3. HT22 cells were transfected with SIRT3 siRNA for 48 h, and then treated with ELA32 (1.5 μM) and OGD/R. A. Ac-PGC-1α was immunoprecipitated using PGC-1α and antiacetylated lysine antibodies. B. NRF1 and TFAM protein expression levels were measured by western blotting. C. ATP level was measured by kits. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group, &&P < 0.01 vs. the scrb group.

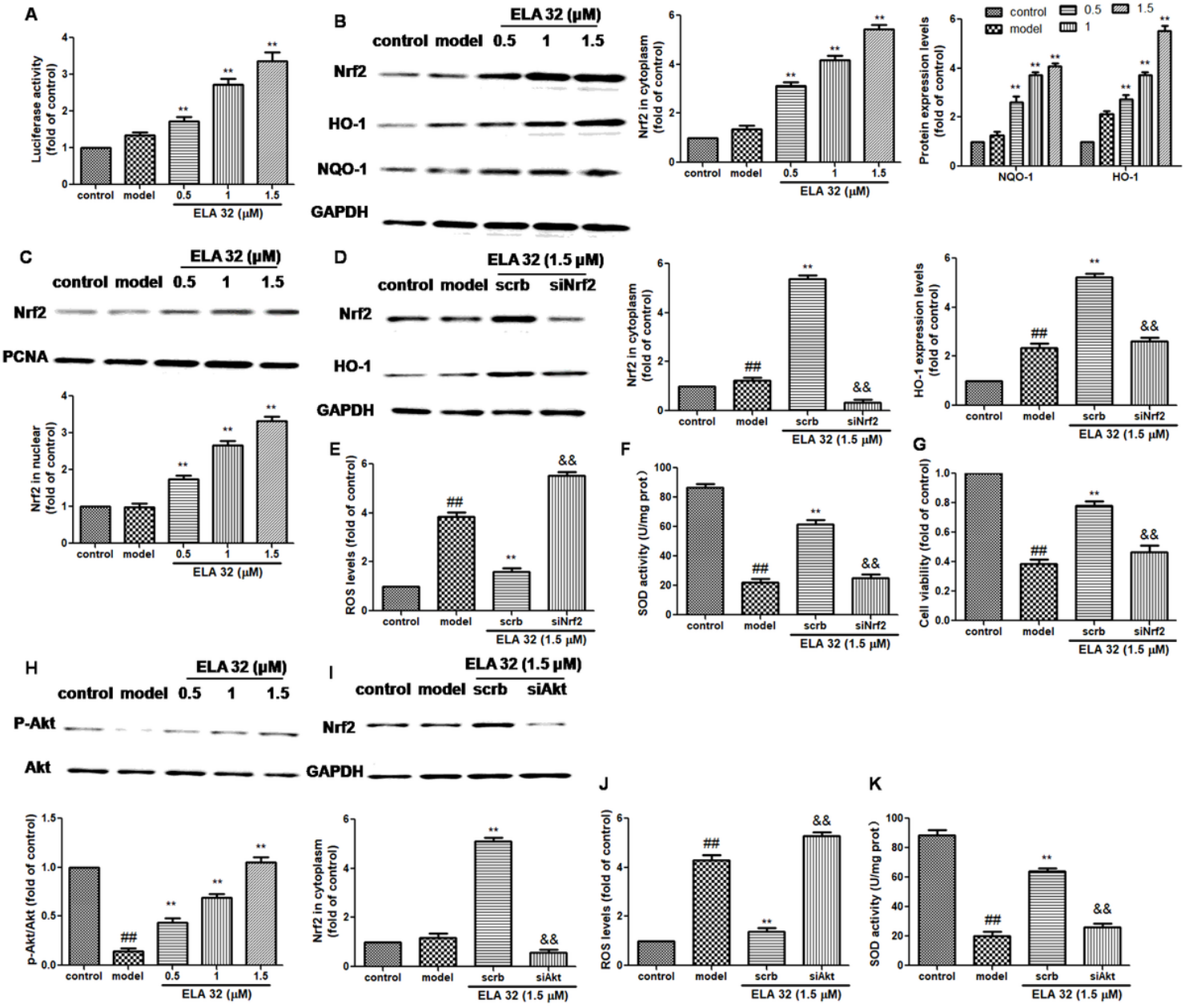

Figure 9

Effects of ELA32 on Nrf2 was through regulating Akt. A. The transcriptional activity of Nrf2/ARE was measured by the ARE luciferase reporter assay. B. Protein expression levels of Nrf2, NQO-1 and HO-1 in cytoplasm. C. Nrf2 expression levels in nuclear. PCNA was sent as the nuclear control. HT22 cells were transfected with Nrf2 siRNA for 48 h, and then treated with ELA32 (1.5 μM) and OGD/R. D. Nrf2 and HO-1 expression levels in cytoplasm after transfection. E. ROS levels after siNrf2 transfection. F. SOD activity after siNrf2 transfection. G. Cell viability after siNrf2 transfection. H. The phosphorylation level of Akt after ELA32 treatments. I. Nrf2 expression levels in cytoplasm after siNrf2 transfection. J. ROS levels after siAkt transfection. K. SOD activity after siAkt transfection. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group, &&P < 0.01 vs. the scrb group.

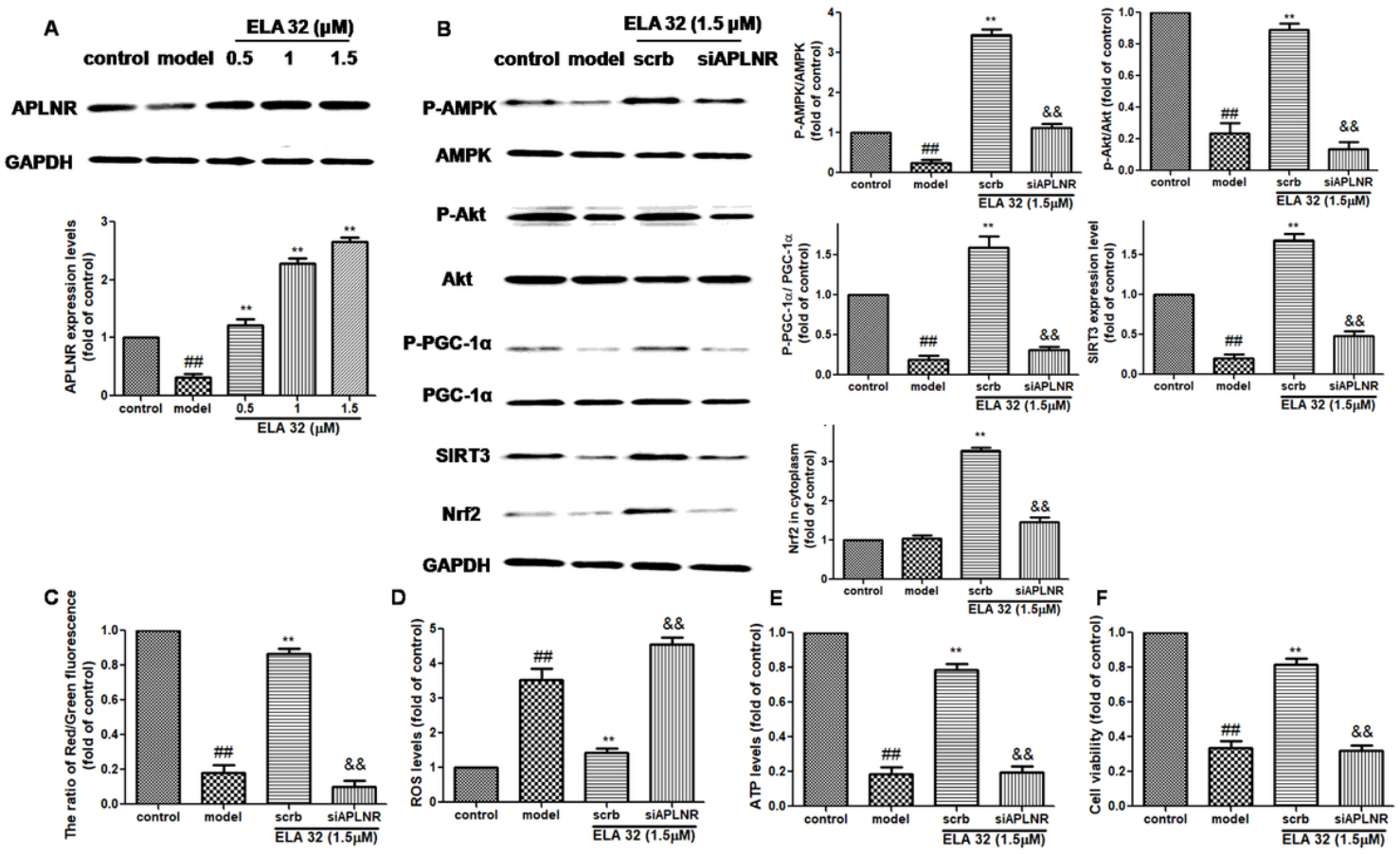

Figure 10

Effects of ELA32 on AMPK and Akt was through regulating APLNR. A. Effects of ELA32 on the protein expression levels of APLNR. B. HT22 cells were transfected with APLNR siRNA for 48 h, and then treated with ELA32 (1.5 μM) and OGD/R. P-AMPK, AMPK, P-Akt, Akt, P-PGC-1α, PGC-1α, SIRT3 and Nrf2 protein expression levels after transfection. C. MMP was measured by JC-1 after transfection. D. ROS levels after transfection. E. ATP levels after transfection. F. Cell viability after transfection. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the control group; **P < 0.01 vs. the model group, &&P < 0.01 vs. the scrb group.

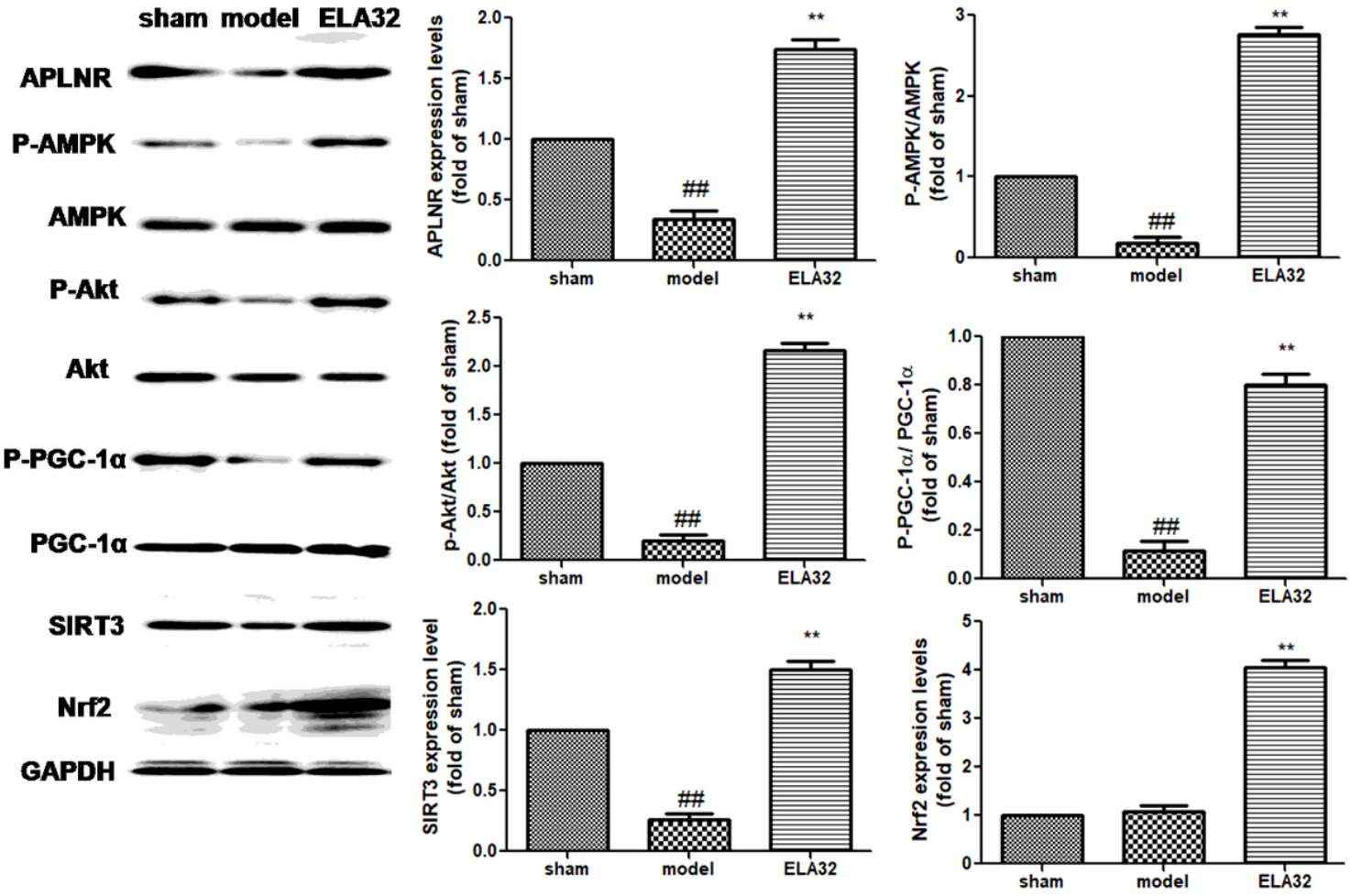

Figure 11

Effects of ELA32 on the APLNR related pathways in brain. Elabela 32 (120 μg/kg) was intracerebral ventricle injected 15 min before reperfusion and given once a day for 3 days after MCAO. Brain tissues were collected and proteins were imaged by Western bloting. Data were expressed as mean ± SD (n≥5). ##P<0.01 vs. the sham group; **P < 0.01 vs. the model group.