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Cryptotanshinone protects hippocampal neurons against oxygen-glucose deprivation-induced injury through the activation of Nrf2/HO-1 signaling pathway

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Abstract

Cryptotanshinone (CPT), a terpenoid isolated from the roots of Salvia miltiorrhiza Bunge, was reported to have neuroprotective effects against cerebral ischemic stroke. However, the exact molecular mechanism underlying its neuroprotective ability remains unclear. The present study aimed to explore the regulatory effects of CPT on oxygen-glucose deprivation/reoxygenation (OGD/R)-induced cell injury in a model of hippocampal neurons. Our results demonstrated that CPT improved cell viability and reduced the lactate dehydrogenase leakage in OGD/R-stimulated hippocampal neurons. In addition, CPT significantly inhibited oxidative stress and apoptosis in hippocampal neurons after OGD/R stimulation. Furthermore, CPT significantly enhanced the nuclear translocation of the nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expression in hippocampal neurons exposed to OGD/R. Moreover, Nrf2 knockdown reversed the anti-apoptotic and anti-oxidant activities of CPT in primary hippocampal neurons exposed to OGD/R. In conclusion, these findings demonstrated that CPT attenuated oxidative stress and neuronal apoptosis after OGD/R injury through the activation of Nrf2/HO-1 signaling pathway in hippocampal neurons. Thus, CPT may be a novel therapeutic agent for cerebral I/R injury.

Keywords: cerebral ischemia/reperfusion (I/R) injury; Cryptotanshinone; hippocampal neurons; neuroprotective; Nrf2/HO-1 pathway; oxidative stress.

Practical Application: Cryptotanshinone may be a novel therapeutic agent for cerebral I/R injury.

1 Introduction

Stroke is a leading cause of death and permanent adult disability all over the world and remains a major challenge to public health (Xie et al., 2018). The treatment for this condition usually involves the restoration of blood flow as quickly as possible (Puyal et al., 2013). However, this can entail secondary injury to the ischemic area, referred to as ischemia/reperfusion injury (Chen et al., 2011). The mechanisms of cerebral ischemia/reperfusion injury remain poorly understood. Previous studies have demonstrated that oxygen-glucose deprivation/reoxygenation (OGD/R) could induce overproduction of reactive oxygen species (ROS) in neuronal cells, and that excessive ROS damaged mitochondrial function, eventually resulting in neuronal apoptosis (Zhao et al., 2014; Zhu et al., 2018). Hippocampal neurons have dopamine nerve terminals that are selectively vulnerable to ischemia. Hence, many neuroprotective treatments for ischemic brain injury rely on these pathologic mechanisms. Traditional Chinese herbal medicine has been described in medicine systems as a neuroprotective treatment associated with ischemic brain injury.

Cryptotanshinone (CPT) is a terpenoid isolated from the roots of Salvia miltiorrhiza Bunge, which have been used in traditional Chinese medicine for treatment of a wide variety of clinical conditions (Chen et al., 2013; Tian & Wu, 2013). CPT, which is a well-known active component of dried roots of Salvia miltiorrhiza Bunge (Danshen), has been reported to possess multiple clinically relevant bioactivities including powerful antibacterial activity, cardiovascular diseases, ischemic stroke (Zhu et al., 2017) and markedly antitumor effects (Vundavilli et al., 2019). CPT also inhibited VEGF-induced angiogenesis by targeting the VEGFR2 signaling pathway (Fei et al., 2017). A previous study showed that CPT exhibited a protective effect against cerebral stroke through inhibition of the PI3K/AKT-eNOS signaling pathway (Zhu et al., 2017). However, the exact molecular mechanism underlying its neuroprotective ability remains unclear. In this study, using the oxygen-glucose deprivation/reperfusion (OGD/R) model of cell ischemia *in vitro*, we investigated the effect of CPT on OGD-induced injury and the underlying mechanism.

2 Materials and methods

2.1 Reagents

CPT, Dimethyl sulfoxide (DMSO) and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). Rabbit antibodies against cleaved caspase-3 (ab32042), Nrf2 (ab89443), lamin B (ab32535), and HO-1 (ab13248) were purchased from Abcam (Cambridge, MA, USA). Anti-Bax (sc-20067), anit-Bcl-2 (sc-492)

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and anti-β-actin (sc-47778) antibodies were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2 Primary hippocampal neuronal cells

Animal experiments conformed to the guidelines issued by the Institute of People's Hospital of Ningxia Hui Autonomous Region (Yinchuan, China) for Laboratory Animals. The present study was performed with approval from by the Animal Ethics Committee of the Institute of People's Hospital of Ningxia Hui Autonomous Region. All surgery was performed under sodium pentobarbital anesthesia (Sigma, St. Louis, MO), and all efforts were made to minimize suffering. Primary culture of hippocampal neurons was isolated and cultured as previously described (Godoi et al., 2013). Briefly, primary hippocampal neurons were prepared from embryonic day 18 (E18) Wistar rat brains. Neurons were plated on poly-D-lysine and laminin coated 6-well dishes at densities of 1×10^6/well as previously described (Xu et al., 2018). Neurons were grown at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air in Neurobasal medium supplemented with B-27, glutamine (0.5 mM), glutamate (25 mM), and 1% penicillin/streptomycin, and then half-replaced twice every week. Culture cells were used after 14 days *in vitro*.

2.3 Oxygen-Glucose Deprivation/Reperfusion (OGD/R) model

The model for oxygen and glucose deprivation/reperfusion (OGD/R) was established using the anaeropack method as described previously (Yang et al., 2018). Briefly, The cells were cultured in glucose-free DMEM and then placed inside a sealed air tight container which contains an anaeropack (Mitsubishi Gas Company, Tokyo, Japan), which resulted in a hypoxic atmosphere by absorbing oxygen and generating carbon dioxide. The cells were maintained in hypoxic conditions at 37 °C for 3 h. Thereafter, the medium was discarded, normal DMEM with glucose was added and culturing continued for 24 h of reoxygenation under normoxic condition to produce OGD/R. The cells cultured in growth culture medium under normoxic condition served as a control.

2.4 Cell viability assay and lactate dehydrogenase release assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli-um bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After treatments, cells were treated with 5 mg/ml MTT (20 μl/well) for 4 h. Then, the medium was discarded and dimethyl sulfoxide (200 ml/well) was added to dissolve the formazan crystals. Absorbance at 490 nm was detected by a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) (Wang et al., 2017).

The release of lactate dehydrogenase (LDH) in the culture medium was determined using a commercially available kit (Jiancheng Biochemical, Nanjing, China), as per manufacturer's protocol. Briefly, the supernatants of each well were transferred into a 96-well microplate, and reaction mixture was added to each well for 30 min at room temperature. Optical density was measured as the LDH levels using a microplate reader (Bio-Tek, Winooski, VT, USA), and all data were represented as folds over control (Chao et al., 2014).

2.5 Measurement of Reactive Oxygen Species (ROS), MDA, SOD and GPx

ROS levels were detected by DCFH-DA assay. Cells were treated with 50 μM DCFH-DA (Sigma, Munich, Germany) and incubated at 37 °C in dark for 30 min. After cells were washed with phosphate buffer solution, the fluorescence intensity was detected by fluorescence spectrophotometer (Bio-Tek, Winooski, VT, USA) with an emission wavelength of 530 nm and an excitation wavelength of 485 nm.

MDA, SOD and GPx levels in primary culture of rat hippocampal neurons were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' protocol.

2.6 Caspase-3 activity

Caspase 3 activity was detected with Caspase 3 activity assay kit (Beyotime) per the manufacturer's instructions. Briefly, hippocampal neurons were lysed and incubated with the substrate Ac-DEVD-pNA (2 mM) at 37 °C for 1 h. Then the absorbance values were read at 405 nm.

2.7 Transfection with short interfering RNAs (siRNAs)

Nrf2 siRNA was designed and manufactured by RiboBio Co. (Guangzhou, China). Neurons cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Experiments using transfected cells were performed 48 h after transfection Scrambled siRNA was used as a control (Liao et al., 2016)

2.8 Western blot assay

For Western blot analysis, 20 μg of total protein of each sample were separated by 10% SDS-PAGE, and transferred onto PVDF membrane at 400 mA for 1.5 h. After transfer, the membrane was blocked with blocking solution, then incubated with the primary antibodies at 4 °C overnight. The blot was then washed with PBST (10 mM NaH_2PO_4, 130 mM NaCl, 0.05% Tween 20) three times, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing three times in PBST, the blot was developed using Pierce ECL Western Blotting Reagents (Thermo Fisher Scientific, Waltham, MA, USA) (Tsung-Chang et al., 2018).

2.9 Statistical analysis

The quantitative data are shown as the mean ± SEM. A one-way analysis of variance followed by a Dunnett's post hoc test was applied for analyzing the multiple comparisons. The statistical analysis was performed using the SPSS 18.0 (SPSS, Chicago, IL), and the statistical significance was set at $P < 0.05$.

3 Results

3.1 CPT improves cell viability in OGD/R-induced hippocampal neurons

To investigate the neuroprotective effects of CPT on hippocampal neurons with OGD/R injury, we first performed cell viability. The obtained results revealed that CPT (2.5-10 μM) did

not affect cell viability (Figure 1A). Thus, these concentrations of CPT were selected in the following experiments. Furthermore, the cell viability of hippocampal neurons induced by OGD/R decreased significantly to 38.5%. However, CPT improved cell viability in hippocampal neurons in response to OGD/R. We further analyzed whether CPT could influence the cell cytotoxicity of hippocampal neurons. As indicated in Figure 1C, CPT dose-dependently reduced OGD/R-induced LDH release in hippocampal neurons.

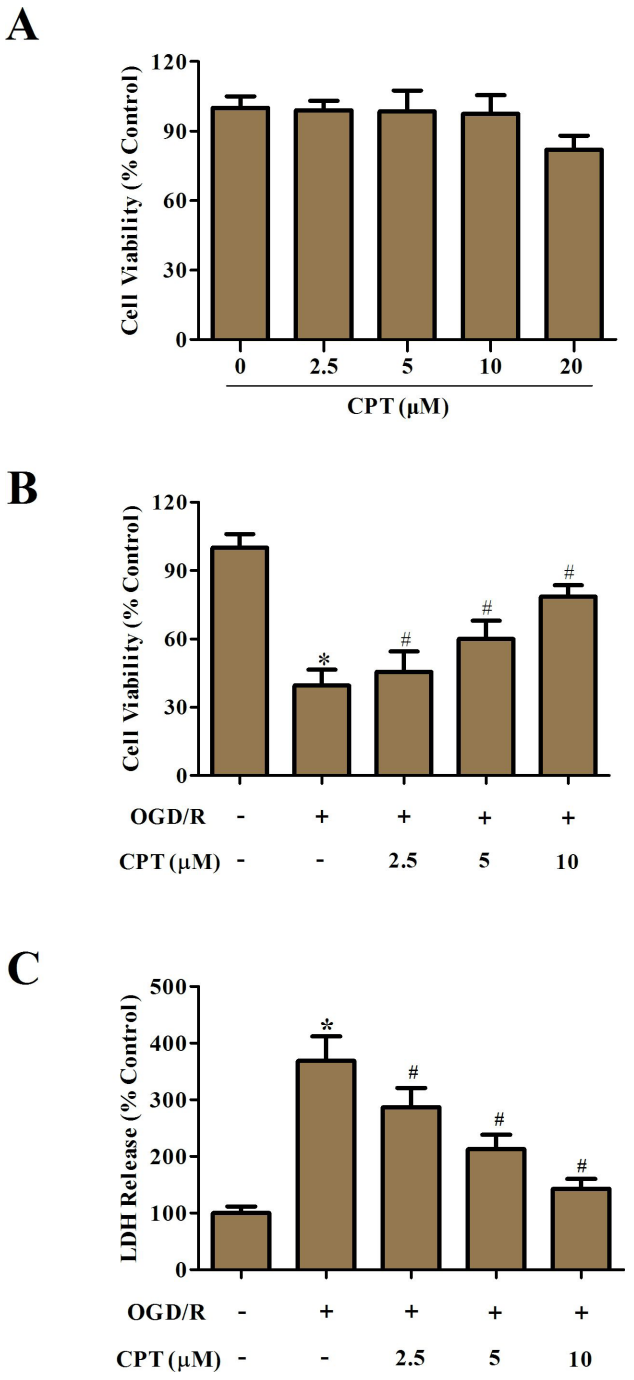

Figure 1. CPT improves cell viability in OGD/R-induced hippocampal neurons. (A) Cell viability of hippocampal neurons after incubation with CPT (0, 2.5, 5, 10, and 20 μM) for 48 h; (B) Cell viability of hippocampal neurons subjected to OGD/R induction with or without the presence of CPT; (C) LDH release of hippocampal neurons subjected to OGD/R induction with or without the presence of CPT; *$P < 0.05$ relative to control cells without OGD/R induction; #$P < 0.05$ relative to OGD/R-induced cells without CPT treatment.

3.2 CPT inhibits OGD/R-induced oxidative stress in hippocampal neurons

The neuroprotective property of CPT is associated with its antioxidant capacity. So we explored the effects of CPT on OGD/R-induced oxidative stress by detecting the relevant biomarkers. The results showed that the ROS levels induced by OGD/R were significantly decreased by CPT (Figure 2A). Significant increase in MDA level (Figure 2B), and decreases in SOD and glutathione peroxidase (GPx) activities (Figure 2C, D) relative to the control levels were observed in OGD/R-induced hippocampal neurons, and the changes were reversed by the administration of CPT. These results demonstrate the anti-oxidative ability of CPT against the oxidative stress induced during OGD/R injury.

3.3 CPT suppresses OGD/R-induced cell apoptosis in hippocampal neurons

We next tested whether CPT affects the cell apoptosis of hippocampal neurons after OGD/R stimulation. The results of western blot assay demonstrated that OGD/R significantly increased the expression levels of Bax and cleaved caspase 3, as well as reduced the expression level of Bcl-2 in hippocampal neurons. However, these effects were reversed by CPT (Figure 3A-D). Furthermore, the caspase-3 activity was higher in OGD/R induced hippocampal neurons. However, 10 μM CPT pretreatment significantly reduced caspase-3 activity in OGD/R-induced hippocampal neurons (Figure 3E). 10 μM CPT treatment only had no effect on Bax, Bcl-2, cleaved caspase 3 expressions and relative caspase-3 activity.

3.4 CPT induces the activation of Nrf2/HO-1 pathway in hippocampal neurons induced by OGD/R

To characterize the underlying mechanism which is responsible for CPT-mediated effect, we investigated the action of CPT on Nrf2/HO-1 pathway in hippocampal neurons. As a result, OGD/R increased the expression of Nrf2 in nuclear fraction and decreased the expression of Nrf2 in cytosolic fraction, whereas, CPT markedly enhanced the activation of Nrf2/HO-1 pathway in OGD/R-stimulated hippocampal neurons (Figure 4A-C). In parallel, OGD/R obviously increased the protein expression of HO-1 in hippocampal neurons, while CPT treatment enhanced these changes elicited by OGD/R (Figure 4A, D). Together, these data demonstrated that CPT promoted Nrf2 nuclear translocation and HO-1 expression in hippocampal neurons induced by OGD/R. However, 10 μM CPT treatment only had no effect on the activation of Nrf2/HO-1 pathway.

3.5 Nrf2 knockdown blocks the effects of CPT against apoptosis

To further evaluate the role of Nrf2/HO-1 pathway, Nrf2 was silenced by si-Nrf2. Western blot analysis showed that the protein expressions of Nrf2 and HO-1 were dramatically decreased

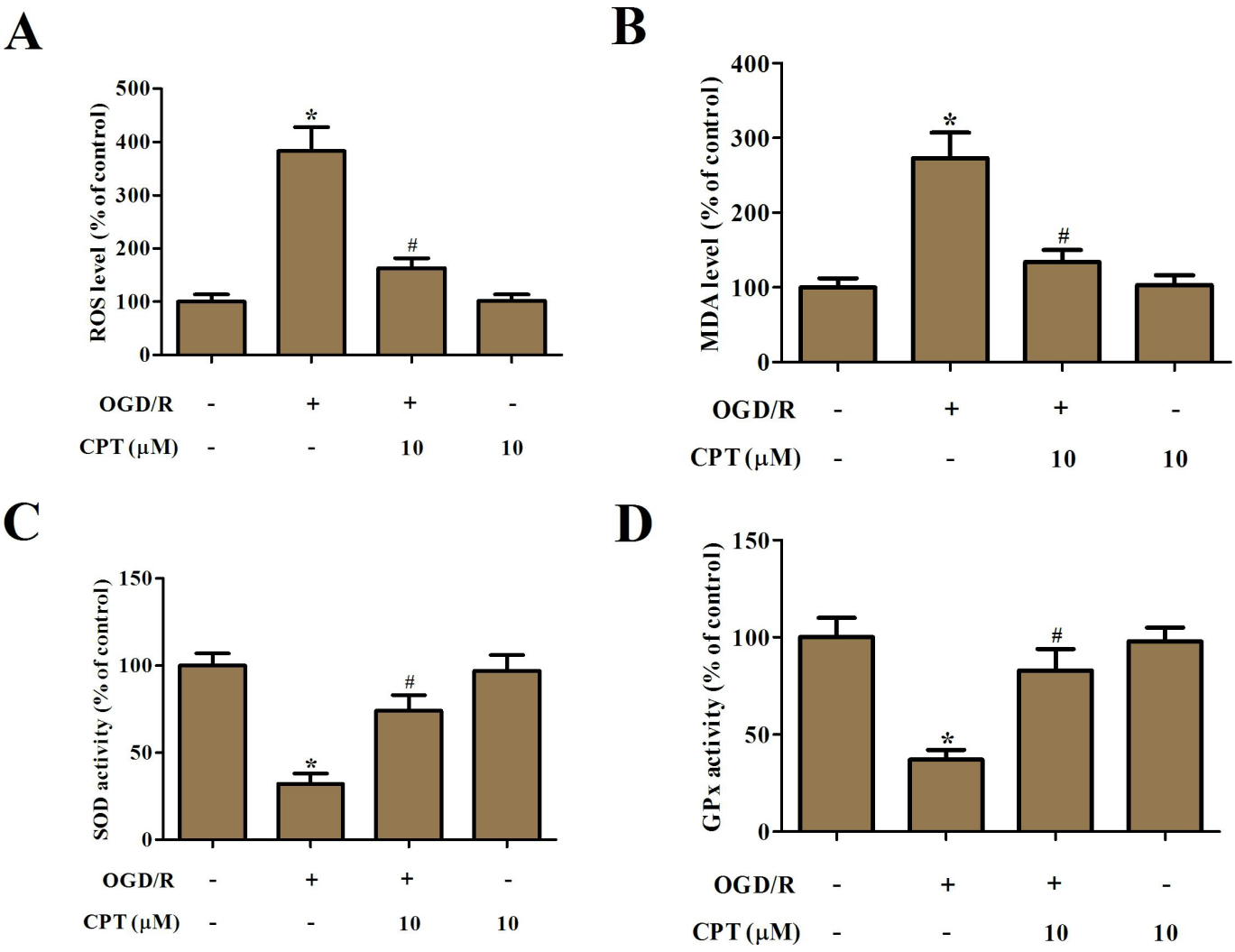

Figure 2. CPT inhibits OGD/R-induced oxidative stress in hippocampal neurons. Hippocampal neurons were subjected to OGD/R induction with or without the presence of CPT. Oxidative stress was evaluated through detecting the levels of ROS and MDA, as well as the activities of SOD and GPx. (A) ROS production; (B) MDA production; (C) SOD activity; (D) GPx activity; *$P < 0.05$ relative to control cells without OGD/R induction; #$P < 0.05$ relative to OGD/R-induced cells without CPT treatment.

after transfection with si-Nrf2 (Figure 5A-C). Furthermore, our data showed that Nrf2 knockdown partially reversed the effects of CPT on cell viability, LDH release and caspase 3 activity in OGD/R-induced hippocampal neurons (Figures 5D-F). The results indicated that the Nrf2/HO-1 pathway was implicated in the neuroprotective effects of CPT.

3.6 Nrf2 knockdown blocks the effects of CPT against oxidative stress

Next, we investigated the role of Nrf2 knockdown on the effects of CPT against oxidative stress by detecting the relevant biomarkers. Administration of CPT markedly reduced the level of ROS and MDA in hippocampal neuron exposed to OGD/R, which was crucially reversed by downregulation of Nrf2 (Figure 6A, B). In parallel, the promotive effect of CPT on SOD and GPx activities was also markedly abolished by Nrf2 silencing in OGD/R-induced hippocampal neurons (Figure 6C, D). Therefore, we concluded that Nrf2 knockdown blocked the effects of CPT against oxidative stress *in vitro*.

4 Discussion

Cerebral ischemia is a common cause of death worldwide, after cardiovascular diseases and cancer, and its prevalence increases with increasing age (Teng et al., 2019). After the onset of brain ischemia, a series of events lead ultimately to the death of neurons (Sutherland et al., 2012). Growing evidence has supported that anti-oxidative, anti-inflammatory and cell protective properties of natural plants and active constituents (Popović et al., 2010; Lee et al., 2010). However, therapies for ischemic brain injury are far from satisfactory and many of these attempts have failed. Therefore, there remains an urgent need to find substances or drugs that can limit or reverse ischemic injury.

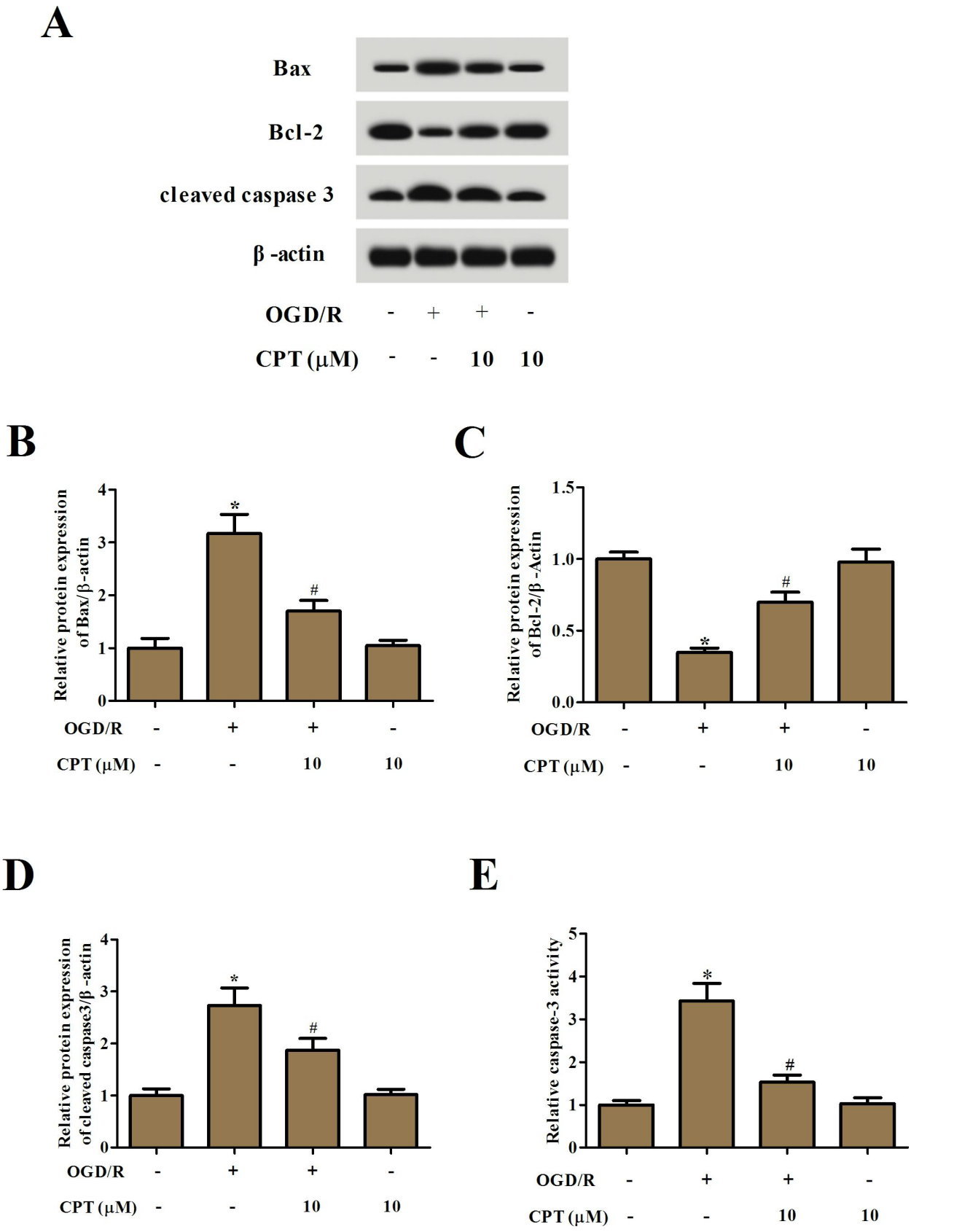

Figure 3. CPT suppresses OGD/R-induced cell apoptosis in hippocampal neurons. (A) The expressions of apoptotic related proteins including Bax, Bcl-2, and cleaved caspase-3 were measured by western blot analysis; (B-D) Quantitation analysis of the western blot analysis for Bax, Bcl-2 and cleaved caspase-3 expression; (E) Caspase 3 activity was detected with Caspase 3 activity assay kit; *$P < 0.05$ relative to control cells without OGD/R induction; #$P < 0.05$ relative to OGD/R-induced cells without CPT treatment.

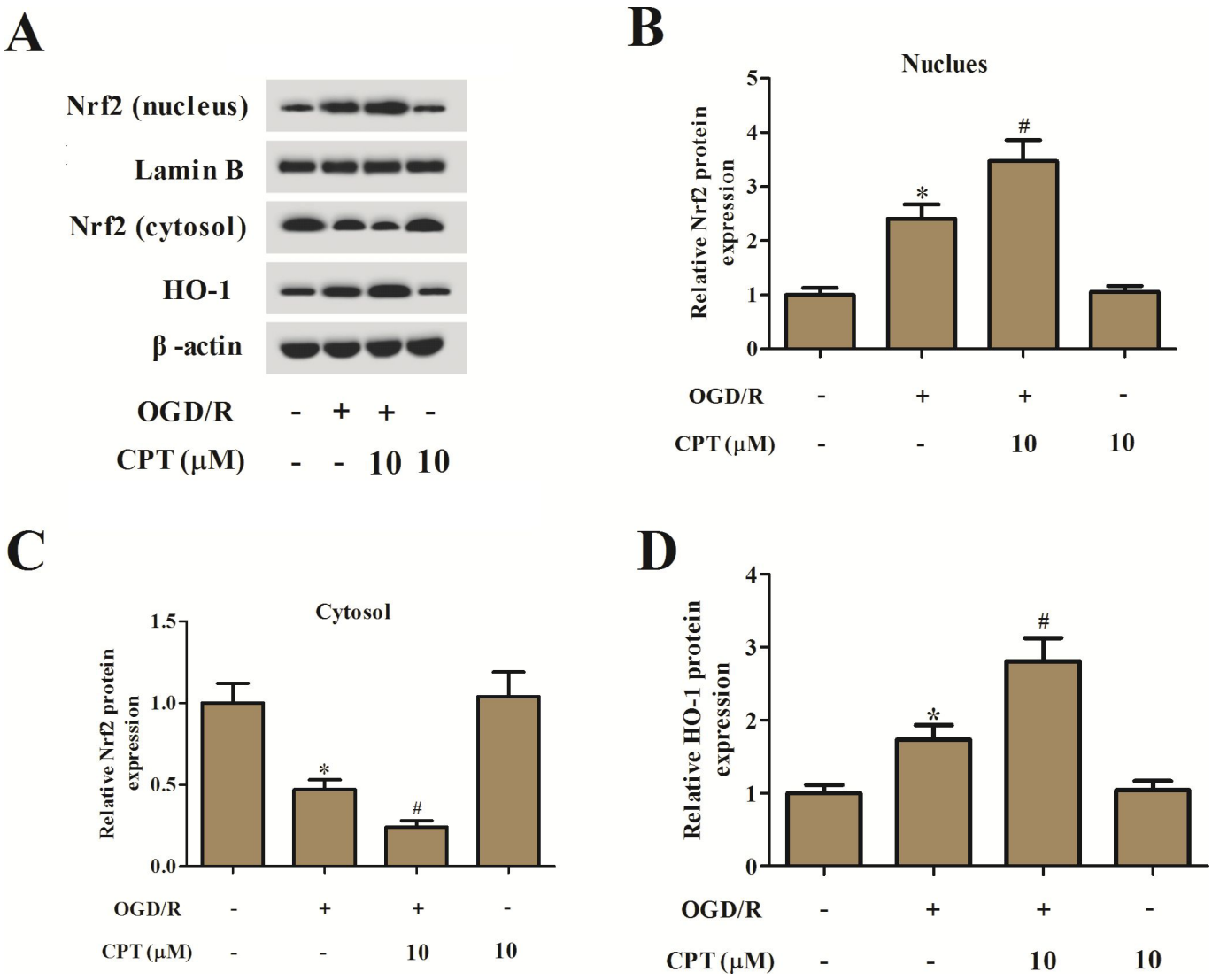

Figure 4. CPT induces the activation of Nrf2/HO-1 pathway in hippocampal neurons induced by OGD/R. (A) The cytosolic extracts and nuclear extracts were prepared from the hippocampal neurons and the expression of Nrf2 and HO-1 were determined by western blot; (B) Quantitation of the western blot analysis for Nrf2 expression in the nucleus; (C) Quantitation of the western blot analysis for Nrf2 expression in the cytoplasm; (D) Quantitation of the western blot analysis for HO-1 expression;*$P < 0.05$ relative to control cells without OGD/R induction; #$P < 0.05$ relative to OGD/R-induced cells without CPT treatment.

Salvia miltiorrhiza Bunge (Danshen) extract could be a promising agent to inhibit thrombosis formation and protect against cerebral ischemia injury (Cao et al., 2016). The present study demonstrated that CPT protects neurons against cell apoptosis processes induced by OGD. These results seem to be in agreement with literature data showing OGD possesses good neuroprotective effects against ischemic brain injury. It has been reported that CPT presented a protective effect against cerebral stroke. However, the exact mechanism of CPT in neuroprotection is poorly understood.

ROS, primarily arising from oxidative cell metabolism, is reported to be related to neuronal damage and death *in vivo* and *in vitro* (Taylor et al., 2005). Excessive ROS production is also one of the mechanisms leading to ischemia/reperfusion-induced cellular injury (Blokhina et al., 2003). The MDA level is one of the most important organic expressions of oxidative stress in various neuronal diseases including ischemia (Siddiqui et al., 2008). Cells also have antioxidant defense mechanisms that involve enzymatic components such as GPx and SOD. SOD and GPx act as endogenous free-radical scavengers, whereas GPx also functions biochemically to reduce lipid hydroperoxides to the corresponding alcohols (Muller et al., 2007). In this study, OGD/R injury in hippocampal neurons caused excessive mitochondrial ROS generation, elevated MDA levels, and reductions in the GPx and SOD activities. After CPT treatment during the reperfusion period, the levels of mitochondrial ROS and MDA were dramatically reduced, simultaneously with the significant increases in SOD and GPx activities. This suggests that neuroprotection is afforded by CPT by reducing the oxidative stress produced under OGD/R conditions.

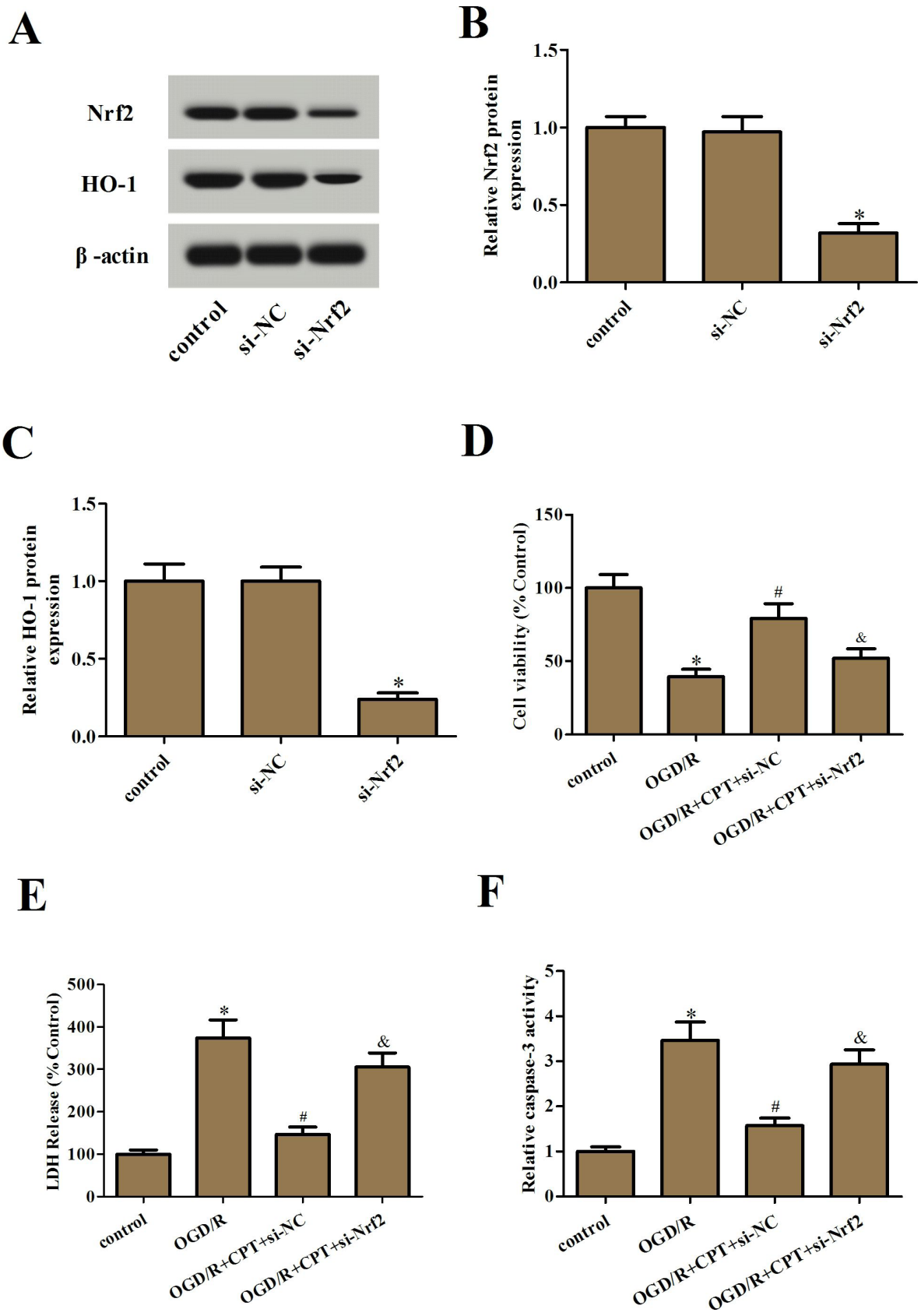

Figure 5. Nrf2 knockdown blocks the effects of CPT against apoptosis. (A) Transfection efficiency was tested using western blot analysis after transfection with si-Nrf2 or control siRNA (si-NC); (B, C) Quantitation analysis of western blot analysis for Nrf2 and HO-1 expression; (D) Cell viability; (E) Lactate Dehydrogenase release; (F) Relative caspase 3 activity; *$P < 0.05$ relative to control cells without OGD/R induction; #$P < 0.05$ relative to OGD/R-induced cells without CPT treatment; &$P < 0.05$ relative to OGD/R-induced cells transfected with si-NC.

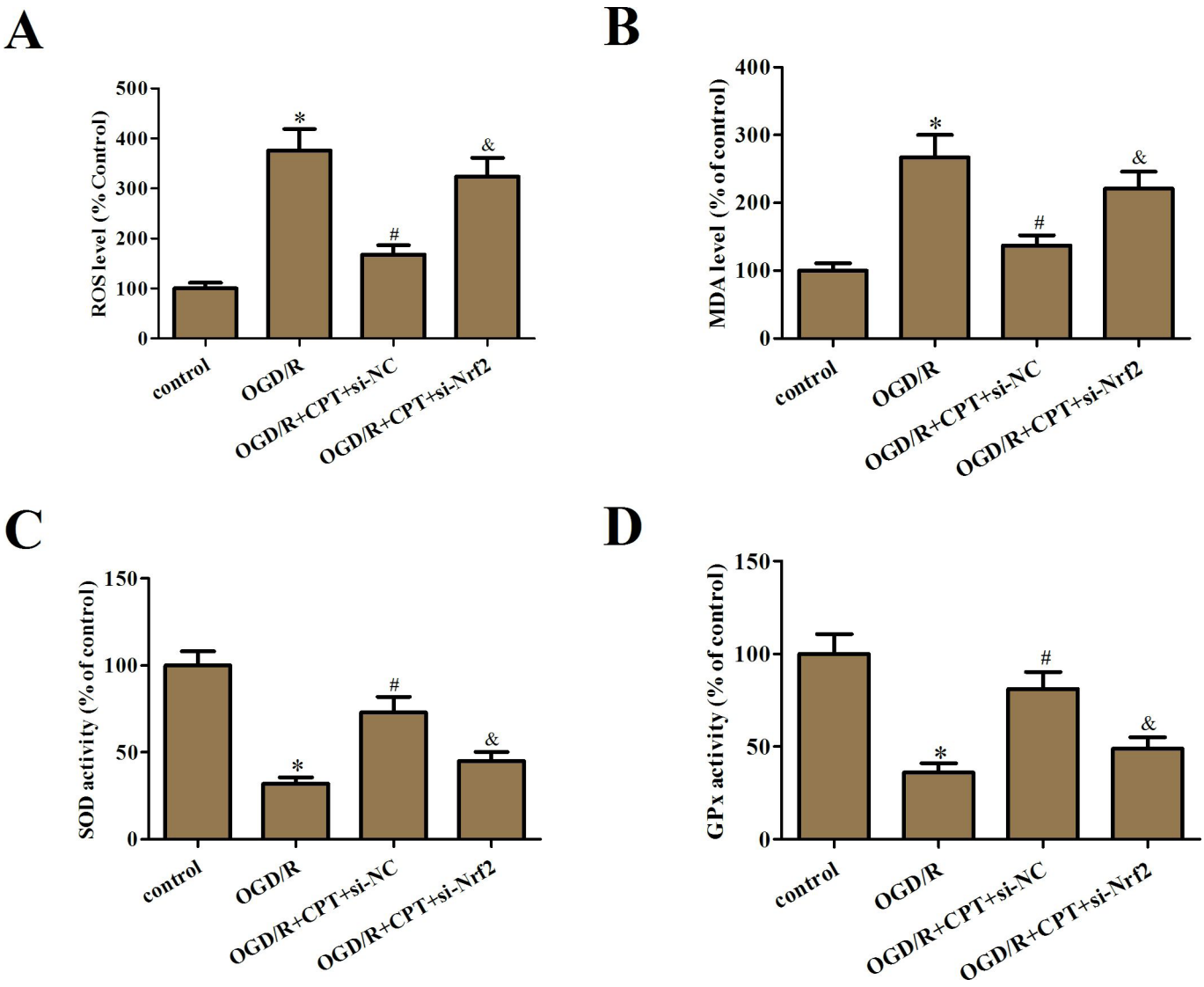

Figure 6. Nrf2 knockdown blocks the effects of CPT against oxidative stress. (A) ROS production; (B) MDA production; (C) SOD activity; (D) GPx activity; *P < 0.05 relative to control cells without OGD/R induction; #P < 0.05 relative to OGD/R-induced cells without CPT treatment; & P < 0.05 relative to OGD/R-induced cells transfected with si-NC.

Nrf2 is an antioxidant transcription factor that plays a key role in resisting oxidative stress caused by various stress environments (Ishimoto et al., 2020). Nrf2 is an endogenous antioxidant defense system and could maintain cellular homeostasis under stress conditions (Fetoni et al., 2015). Loss of Nrf2 increases the size of cerebral infarct and neurological deficits after cerebral ischemia/reperfusion injury (Ashabi et al., 2015). In addition, a study also indicated that activation of Nrf2 improved recovery of the hematopoietic system after radiotherapy (Kim et al., 2014). The Nrf2/HO-1 pathway is a crucial pathway that is involved in oxidative stress (Vomhof-Dekrey & Picklo, 2012). Dutta et al. reported that activation of the Nrf2/HO-1 pathway contributes to early recovery of the gastrointestinal and hematopoietic systems and increased survival following whole body irradiation in mice (Dutta, Gupta, & Kalita, 2015). Consistent with these reports, in this study, we found that CPT enhanced the activation of Nrf2/HO-1 pathway in OGD/R-induced hippocampal neurons. We further demonstrated that the Nrf2/HO-1 pathway was implicated in the neuroprotective effects of CPT.

In summary, we explored the role of CPT in an *in vitro* OGD/R model in primary hippocampal neurons. The results showed that CPT attenuated OGD/R-caused oxidative stress, cell damage, and apoptosis in hippocampal neurons. CPT enhanced the activation of the Nrf2/HO-1 pathway in OGD/R-induced hippocampal neurons. Transfection with si-Nrf2 abolished the protective effect of CPT. These data indicated that CPT protected hippocampal neurons from OGD/R-induced cell damage through the activation of Nrf2/HO-1 pathway.

Availability of data and material

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

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