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Carvedilol, a third-generation β-blocker prevents oxidative stress-induced neuronal death and activates Nrf2/ARE pathway in HT22 cells

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ABSTRACT

Carvedilol, a nonselective β-adrenoreceptor blocker with pleiotropic activities has been shown to exert neuroprotective effect due to its antioxidant property. However, the neuroprotective mechanism of carvedilol is still not fully uncovered. Nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway is an important cellular stress response pathway involved in neuroprotection. Here we investigated the effect of carvedilol on oxidative stress-induced cell death (glutamate 2 mM and H_2O_2 600 μM) and the activity of Nrf2/ARE pathway in HT22 hippocampal cells. Carvedilol significantly increased cell viability and decreased ROS in HT22 cells exposed to glutamate or H_2O_2. Furthermore, carvedilol activated the Nrf2/ARE pathway in a concentration-dependent manner, and increased the protein levels of heme oxygenase-1(HO-1) and NAD(P)H quinone oxidoreductase-1(NQO-1), two downstream factors of the Nrf2/ARE pathway. Collectively, our results indicate that carvedilol protects neuronal cell against glutamate- and H_2O_2-induced neurotoxicity possibly through activating the Nrf2/ARE signaling pathway.

1. Introduction

Progressive oxidative stress is a major event that precedes neuronal death in neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease. Oxidative stress occurs when the antioxidant defense systems are exceeded by free radical production, resulting in oxidative damage of various cellular components [1]. The majority of free radicals originate from the mitochondrial respiratory chain, where an inefficient electron transfer to oxygen during oxidative phosphorylation and ATP production leads to local overproduction of reactive oxygen species (ROS) [2,3]. Oxidative damage emerges when ROS are overproduced accompanying with a reduction in the endogenous anti-oxidative defense. ROS are also recognized to be related to the aging process [4].

The nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway is critical in regulating endogenous antioxidants and phase II detoxification enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO-1). The importance of Nrf2 in protecting cells against toxins and in pathological conditions characterized by enhanced oxidative stress has been clearly demonstrated by using siRNA methodologies and Nrf2 knockout mice [5]. Activating the Nrf2/ARE pathway might be an attractive approach for the therapy of neurodegenerative diseases [5–7].

Carvedilol (Fig. 1), is a nonselective β-adrenoreceptor blocker with multifunctions, such as the blockade of α1-adrenoceptor, the antagonism of NMDA receptors and the inhibition of calcium channel [8–11]. Carvedilol exerts neuroprotective effects in animal models of transient focal stroke [8] and in *in vitro* toxic models induced by aluminium [12], sodium nitroprusside [14] and transthyretin [13]. These neuroprotective effects of carvedilol have been attributed partly to its free radical scavenging and metal chelating properties [15]. However, the mechanism of anti-oxidative stress of carvedilol remains obscure. Given Nrf2/ARE signaling pathway plays an important role in oxidative stress-induced neurotoxicity [5], we hypothesized that the activation of the Nrf2/ARE pathway might be involved in the neuroprotective effects of carvedilol.

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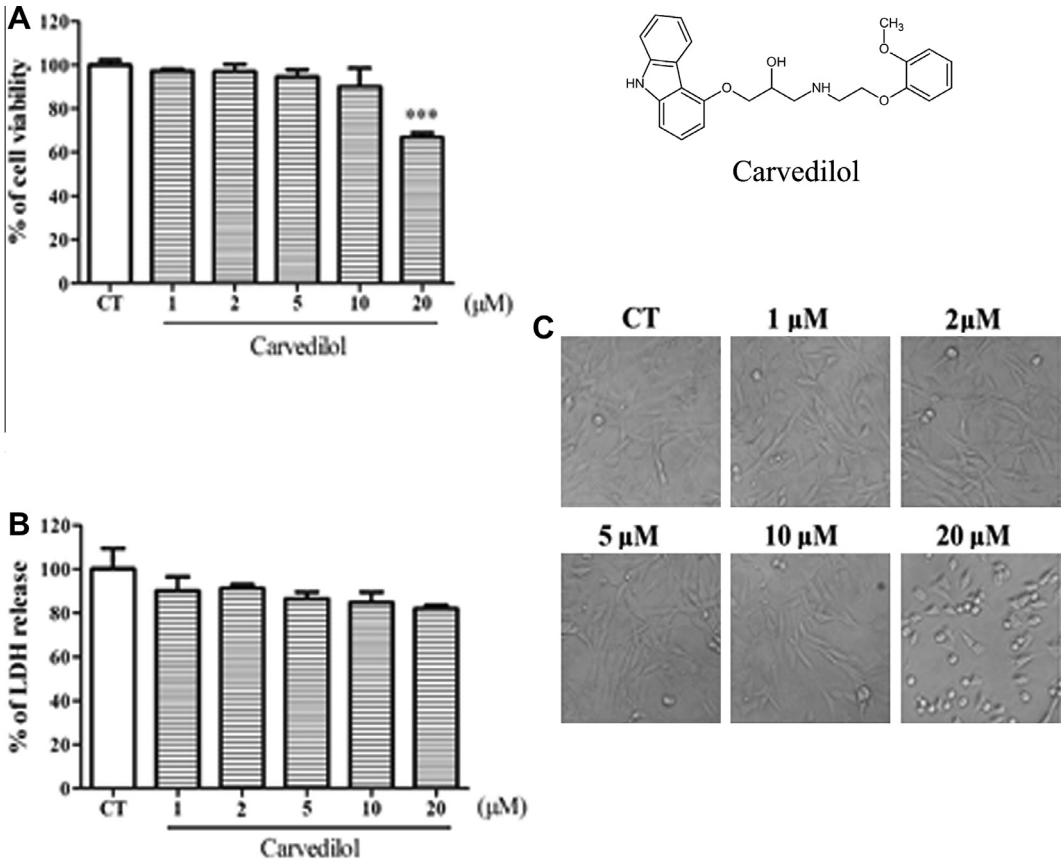

Fig. 1. Effects of carvedilol on the cell viability of HT22 cells. Treatment of HT22 cells with various concentrations for 24 h. (A/B) Cells viability and cytotoxicity were determined by using the MTT and LDH assays respectively. (C) Cells were pretreated with carvedilol at various concentrations as indicated for 24 h, and then were photographed under phase-contrast microscope. One-way ANOVA followed by Tukey's test. Data are presented as means ± s.d. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* control group.

The purpose of the present study was to investigate the effects of carvedilol on oxidative stress-induced neuronal death and to explore the roles of the Nrf2/ARE pathway played in the neuroprotection of carvedilol in HT22 cells, an immortalized mouse hippocampal cell line.

2. Materials and methods

2.1. Materials

Carvedilol (>98% purity) was purchased from Meilun Biotechnology (Dalian, China). Trypsin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dichlorofluorescein diacetate (H_2DCF-DA) were purchased from Sigma–Aldrich (Shanghai, China). Glutamate and H_2O_2 were purchased from Research Biochemicals International (MA, USA). Lipofectamine 2000 was from Invitrogen Biotechnology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (NY, USA). Lactate dehydrogenase (LDH) assay kit was from Jiancheng Biochemical Company (Nanjing, China). The Dual-luciferase Reporter Assay System was bought from Promega, USA. The ARE-driven luciferase reporter plasmid pGL6-ARE and the Renilla luciferase pRL-TK vector were gifted by Dr. Siwang Yu (Peking University). Carvedilol stock solution (100 mM) was in DMSO and kept at −20 °C.

2.2. Cell culture and treatment

HT22 mouse hippocampal neuronal cells were maintained in DMEM supplemented with 10% (v/v) FBS and incubated at 37 °C under 5% CO_2. To study the protective effects of carvedilol on glutamate- or H_2O_2-induced neuronal death, cells were seeded in 96-well plates (10,000 cells/well), and 3 wells were used for each treatment group. The fresh solution of carvedilol (100 mM in DMSO) was diluted in DMEM supplemented with 10% (v/v) FBS immediately before adding to each well at the desired final concentrations. For the experiments, cells were pre-incubated with various concentrations of carvedilol for 30 min, and followed by treatment of glutamate or H_2O_2 for 24 h. Control group was treated with 0.1% (v/v) DMSO as vehicle control.

2.3. MTT assay and LDH release assay

Cell viability was determined by MTT assay as well as the LDH assay. The release of LDH in the culture medium was determined using a commercially kit. Briefly, after treatment, 20 μL supernatant per well was transferred into a 96-well microplate to determine LDH levels according to the manufacturer's instructions before adding MTT. Optical density was measured using a microplate reader (Bio-Tek, USA) at 405 nm. For the MTT assay, MTT (5 mg/mL) 10 μL was then added to each well and the mixture was incubated for 2 h at 37 °C. MTT reagent was then replaced with DMSO (100 μL per well) carefully to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 570 nm using a microplate reader. Results were expressed as the percentage of the absorbance of control cells, which was set as 100%.

2.4. Morphologic changes

HT22 cells (5×10^5 cells/well) were cultured in 6-well plates for 24 h and then exposed to carvedilol at various concentrations for

24 h, morphologic changes were observed by phase-contrast microscopy and the images of cells were taken by a digital camera (Olympus, Japan).

2.5. Measurement of ROS

Intracellular ROS formation was measured by fluorescence using H_2DCF-DA. Briefly, after treatment, cells were washed and then stained with 10 μM H_2DCF-DA in serum-free medium for 30 min at 37 °C in the dark. The cells were photographed using a fluorescence microscope (Olympus, Japan).

2.6. Estimation of intracellular GSH

A GSH assay kit (48T, Nanjing Jiancheng, China) was used to measure intracellular GSH concentration. By reacting with dithiobisnitrobenzoicacid, reduced glutathione (GSH) could form a yellow compound, which is quantifiable at 405 nm and reflect the content of the reduced GSH indirectly. In brief, after treatment, whole-cell lysate was prepared according to manufacturer's instructions. All GSH values were normalized to per μg protein of each sample and the levels of GSH measured in control conditions were set as the 100% values.

2.7. Transient transfection and ARE-luciferase assay

The Dual-luciferase Reporter Assay System (Promega, USA) was used to determine ARE-luciferase activity in transiently transfected HT22 cells. HT22 cells were seed in 48-well plate at density of 3×10^4 per well, and triple wells were used for each groups. The ARE-driven luciferase reporter plasmid pGL6-ARE and the Renilla luciferase pRL-TK vector, used for normalization, were co-transfected into HT22 cells. Each well of cells was transfected with 200 ng of pGL6-ARE and 20 ng pRL-TK using lipofectamine 2000 according to the manufacturer's instructions. After transfection and treatment, the cells lysates were prepared with passive lysis buffer, and the firefly and Renilla luciferase activities were measured using a luminometer according to the manufacturer's instructions. Relative firefly luciferase activity was normalized to Renilla luciferase activity.

2.8. Western blot analysis

Western blotting analysis was performed as previously described [16]. Briefly, proteins were harvested in a cell lysis buffer. Equal amounts of lysate protein (20 μg/lane) were subjected to SDS–PAGE with 10% polyacrylamide gels and electropho-retically transferred to nitrocellulose membranes. Nitrocellulose blots were first blocked with 3% bovine serum albumin (BSA) in PBST buffer (PBS with 0.01% Tween 20, pH 7.4), and incubated overnight at 4 °C with primary antibodies (Table 1) in PBST containing 1% BSA. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibodies, and detected by the enhanced chemiluminescence technique.

2.9. Statistical analysis

The data were presented as the means ± s.d. Statistical analyses between two groups were performed by unpaired Student's *t*-test. Differences among groups were tested by one-way analysis of variance (ANOVA). Following ANOVA analyses, the Tukey's test was used and $p < 0.05$ was accepted to be statistically significant.

3. Results

3.1. Effects of carvedilol on cell viability and morphology of HT22 cells

To find out the concentrations that do not induce cell toxicity, HT22 cells were treated with various concentrations of carvedilol for 24 h, cell viability and cytotoxicity were monitored by the MTT assay and LDH assay respectively (Fig. 1A and B). In MTT assay, carvedilol slightly reduced MTT reduction at 20 μM (Fig. 1A). But in LDH assay, no significant difference was found among groups treated with or without carvedilol (0–20 μM) (Fig. 1B). The images showed that carvedilol (20 μM) slightly inhibited the cell proliferation and also induced morphological changes (Fig. 1C).

3.2. Effects of carvedilol on glutamate- and H_2O_2-induced cytotoxicity

To investigate the neuroprotective effects of carvedilol against glutamate- and H_2O_2-induced oxidative damage, HT22 cells were pretreated with various concentrations of carvedilol for 30 min before exposed to of glutamate (2 mM) or H_2O_2 (600 μM). After 24 h, the cell viability was assayed by the MTT assay. The results showed that glutamate (2 mM) or H_2O_2 (600 μM) significantly reduced cell viability. Carvedilol (3–20 μM) significantly prevented glutamate- or H_2O_2-induced neurotoxicity (Fig. 2A and B), and the best neuroprotection of carvedilol was found at 10 μM (almost fully preventing the damage). Therefore, 10 μM of carvedilol was selected for the following experiments.

3.3. Effects of carvedilol on glutamate- and H_2O_2-induced ROS accumulation and glutamate-induced GSH release in HT22 cells

Previous studies showed that the toxicity of glutamate and H_2O_2 were mediated through the production of ROS [17,18] and carvedilol can quench the ROS production [10]. Therefore, we investigated whether carvedilol blocked glutamate- or H_2O_2-induced oxidative stress and increased the anti-oxidative defense system of HT22 cells. Cellular oxidative stress was determined by H_2DCF-DA staining, a ROS probe [19]. Glutamate and H_2O_2 significantly increased the intracellular production of ROS, and carvedilol significantly reduced glutamate- and H_2O_2-induced ROS production (Fig. 3A). Previous report demonstrated that glutamate potently depleted endogenous GSH level [20]. To further confirm whether the protection of carvedilol against oxidative damage is associated with the reversion of GSH depletion induced by glutamate, we evaluated the effect of carvedilol on the level of GSH in glutamate-treated cells (Fig. 3B). Our results showed that glutamate depleted the intracellular GSH which could not be reversed by carvedilol in HT22 cells.

Table 1
Summary of primary antibodies and their dilution used in the experiments.

Antibody	Species	Source	Dilution
anti-phospho Nrf2	Rabbit, monoclonal	Epitomics, Burlingame CA, USA	1:1000
anti-HO-1	Rabbit, polyclonal	Santa Cruz Biotechnology Inc. CA, USA	1:1000
anti-NQO-1	Rabbit, monoclonal	Epitomics, Burlingame CA, USA	1:1000
anti-tubulin	Mouse, polyclonal	Sigma-Aldrich, St. Louis, MO, USA	1:10,000

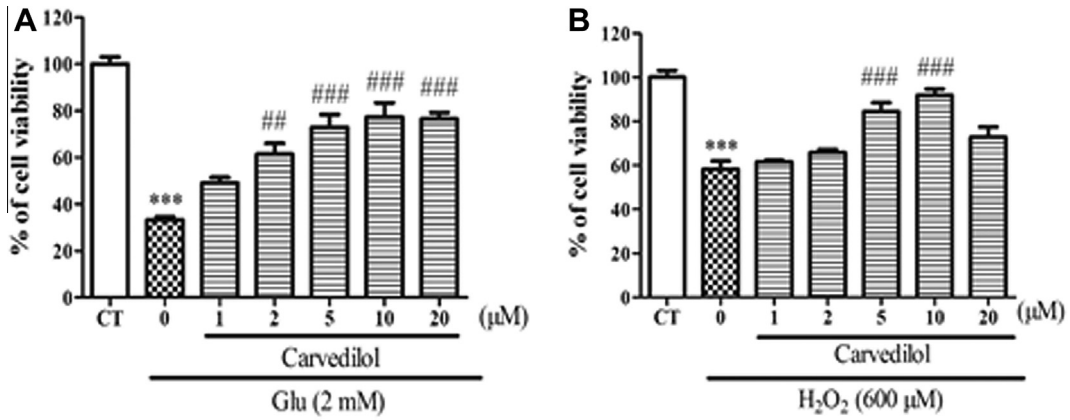

Fig. 2. Carvedilol alleviated glutamate- and H_2O_2-induced neuronal cell death in HT22 cells. Cells were pre-treated with carvedilol at various different concentrations as indicated for 30 min, and then expose to glutamate (Glu, 2 mM) (A) or H_2O_2 (600 μM) (B) for 24 h. Cell viability was assayed by MTT assay. Similar results were obtained in at least three independent experiments. One-way ANOVA followed by Tukey's test. Data are presented as means ± s.d. $^{***}P < 0.001$ *vs.* control group; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs.* glutamate- (A) or H_2O_2-treated (B) groups.

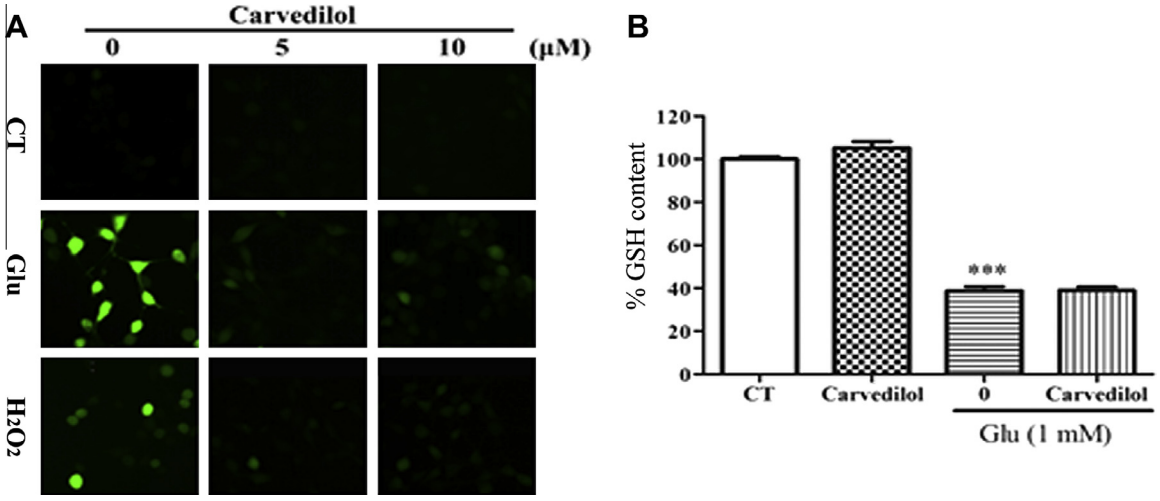

Fig. 3. Carvedilol inhibits glutamate- and H_2O_2-induced ROS formation but not GSH depletion in HT22 cells. (A) Carvedilol significantly inhibits glutamate (Glu, 2 mM) and H_2O_2 (600 μM)-induced production of intracellular ROS. Cells were pretreated with/without carvedilol (5–10 μM) for 30 min, and then exposed to 2 mM glutamate or 600 μM H_2O_2 for 12 h followed by incubation with 10 μM H_2-DCF-DA for 30 min. Cells were photographed using a fluorescence microscope (×200). (B) Intracellular GSH was depleted by Glutamate. Cells were pretreated with carvedilol for 30 min then incubation with glutamate (2 mM) for 12 h. The levels of GSH measured in control conditions were set as the 100% values. $^{***}P < 0.01$ *vs.* control group.

3.4. Effects of carvedilol on Nrf2/ARE pathway in HT22 cells

Nrf2/ARE signaling pathway has been reported to be a key pathway by which regulating a battery of endogenous cytoprotective genes, including those encoding for both antioxidant- and anti-inflammatory proteins, such as HO-1 and NQO-1 [5,6]. To further investigate the effects of carvedilol on oxidative stress, ARE-luciferase assay was used to evaluate the activity of the Nrf2/ARE pathway. As shown in Fig. 4A, similar to the effect of tBHQ, an Nrf2 activator [21], carvedilol significantly increased the activity of luciferase. Afterwards, we investigated the effect of carvedilol on the Nrf2-antioxidative system, the level of p-Nrf2, NQO-1 and HO-1 proteins were examined by western blotting. As shown in Fig. 4B and C, the levels of p-Nrf2, HO-1 and NQO-1 proteins were significantly increased by carvedilol.

4. Discussion

To the best of our knowledge, this is the first report to show that carvedilol significantly attenuated glutamate- and H_2O_2-induced neurotoxicity by decreasing the production of ROS but failed to reverse glutamate-induced depletion of intracellular GSH level in HT22 cells. Meanwhile, carvedilol significantly activated the Nrf2/ARE pathway and then up-regulated the expression of HO-1 and NQO-1.

It is known that carvedilol plays a pivotal role in cardiovascular disease, especially in the aspect of anti-oxidative stress, but the mechanism is still unclear. In neurodegenerative diseases, progressive oxidative stress is a major disastrous event that precedes neuronal death. Neuronal loss is the ultimate outcome in a mass of neurodegenerative diseases. In AD, local oxidative stress surrounding plaques attributes to long lasting toxicity and selective neuronal death [22]. Therefore, antioxidant therapy should be an attractive strategy against neuronal loss [5,6,23]. In this study, we found that glutamate- and H_2O_2-induced cell death and carvedilol significantly attenuated the cytotoxicity of glutamate and H_2O_2 in a concentration-dependent manner, supporting that carvedilol has antioxidant properties [24]. To further investigate the antioxidative properties of carvedilol, we explored the effects of carvedilol on glutamate- and H_2O_2-induced ROS accumulation. As shown in Fig. 3A, carvedilol decreased H_2DCF-DA fluorescence

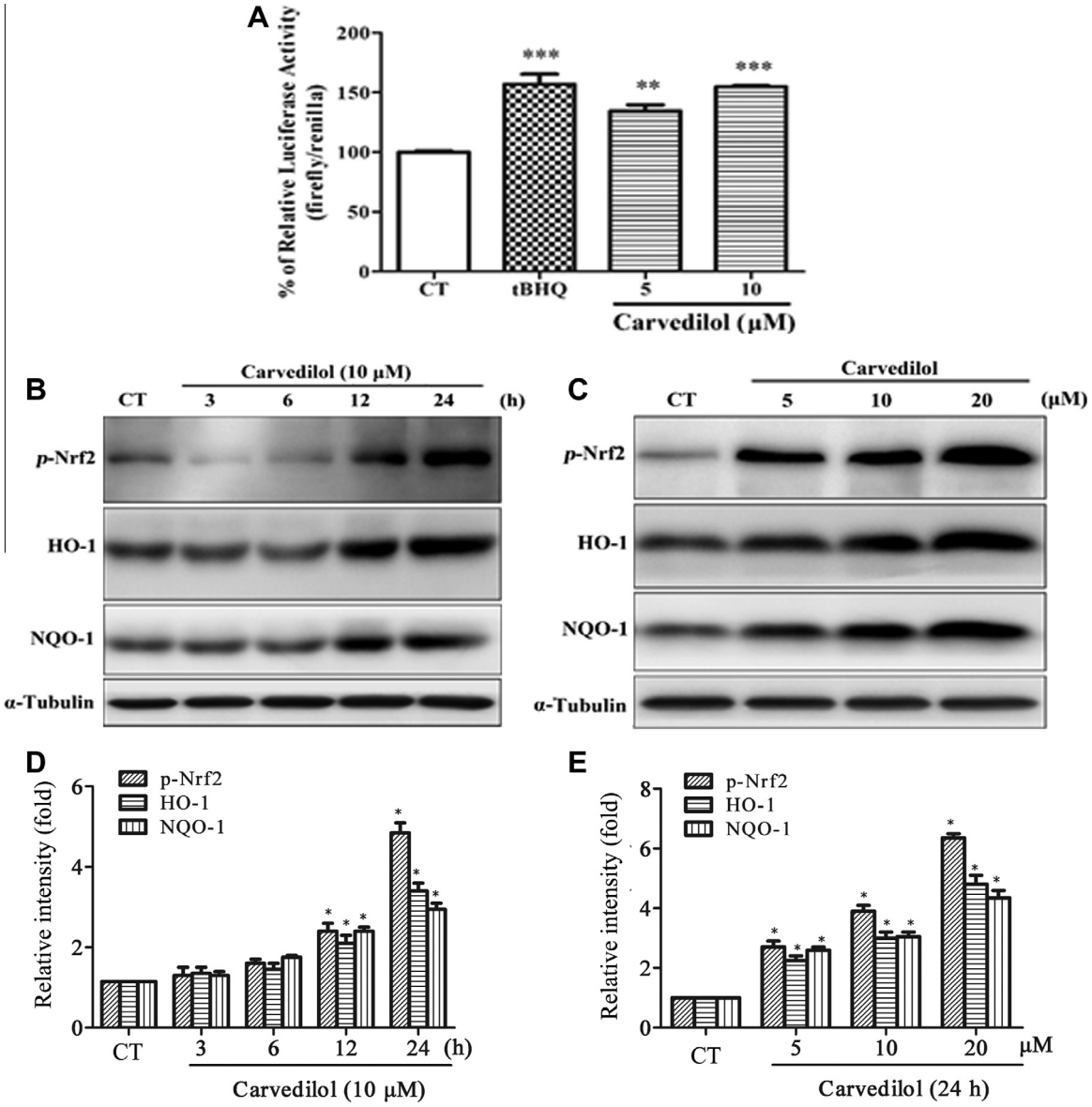

Fig. 4. Effects of carvedilol on the Nrf2/ARE/HO-1 pathway in HT22 cells. (A) The effects of carvedilol on the ARE pathway evaluated by ARE-luciferase assay; (B and D) The time-dependent effects of carvedilol (10 μM) on the Nrf2-antioxidative system, the level of Nrf2, NQO-1 and HO-1 proteins were examined by western blotting in HT22 cells; (C and E) The dose-dependent effects of carvedilol on the Nrf2-antioxidative system. The levels of Nrf2, NQO-1 and HO-1 proteins were examined by western blotting in HT22 cells for 24 h. One-way ANOVA followed by Tukey's test. Data are presented as means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* control groups.

intensity which was induced by glutamate and H_2O_2, indicating that carvedilol possesses potent anti-oxidative stress property. Meanwhile, we examined the effects of carvedilol on glutamate-induced intracellular GSH depletion. It was surprising that carvedilol did not reverse glutamate-induced intracellular GSH depletion. Previous study demonstrated that α-tocopherol, a strong antioxidant, prevents cells from glutamate toxicity without affecting intracellular GSH depletion induced by glutamate [20], suggesting that the protection of carvedilol might also be GSH-independent.

Oxidative damage is a complex process, which may involve DNA adducts, lipid peroxidation and protein modifications. Nrf2, a basic leucine zipper transcription factor, is known to drive transcription of all kinds of genes involved in combating not only products of oxidation (hydroxyl radicals or malondialdehyde, protein and DNA adducts from carbonyls) but also oxygen radicals [25]. It is clear that cells which transcriptionally activated Nrf2 could be resistant to oxidative insult by various mechanisms. Therefore, Nrf2 is an attractive target for prevention of neurodegeneration induced by multifaceted response [5,6]. Besides, Nrf2 is an important regulator of HO-1 protein and ARE-activated gene expression. Under physiological conditions, Nrf2 binds to Kelch-like ECH-associated protein-1 (Keap1). In oxidative stress, Nrf2 is released from Keap1 and quickly translocated to the nucleus, where it binds to ARE sequences resulting in transcriptional activation of antioxidant genes such as thioredoxin-1, HO-1 and NQO-1 [26]. In this study, we found that carvedilol activated the Nrf2/ARE pathway and increased the levels of p-Nrf2, HO-1 and NQO-1, suggesting that the Nrf2/ARE pathway might be involved in the antioxidative capability of carvedilol.

Taken together, our results indicate that carvedilol exerts strikingly protective effects against glutamate and H_2O_2 cytotoxicity in HT22 cells through activating the Nrf2/ARE pathway, which might be helpful for extending the usage of carvedilol for oxidative stress-associated diseases, such as AD.

Conflict of interests

The authors have no conflict of interest.

Acknowledgments

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