Cytoprotective effects evaluation of a novel danshensu derivative DEX-018 against oxidative stress injury in HUVECs

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Ischemic heart disease (IHD) is one of the most common cardiovascular diseases with high morbidity and mortality. Danshensu (DSS) is widely used in the treatment of coronary heart disease. In this study, the carboxy group of DSS was esterified with edaravone to synthesize the novel DSS derivative DEX-018 to achieve a synergistic protective effect and overcome the structural deficiency of DSS. The pharmacological effect of DEX-018 against tert-butyl hydrogen peroxide (t-BHP) induced oxidative damage in human umbilical vein endothelial cells (HUVECs) was evaluated. The results demonstrated that pretreatment with DEX-018 significantly increased cell viability and superoxide dismutase (SOD) activity and decreased the lactate dehydrogenase (LDH) leakage rate, malondialdehyde (MDA) level and intracellular reactive oxygen species (ROS) level. In addition, DEX-018 inhibited cell apoptosis and reversed the expression of apoptosis-related proteins (Bcl-2, Bax, and caspase-3) in HUVECs stimulated by t-BHP. Further study on the mechanism of DEX-018 revealed that the expression of p-Akt and p-extracellular signal-regulated kinase 1/2 (ERK1/2) was increased, which suggested that DEX-018 may protect HUVECs against t-BHP induced oxidative injury via the Akt and ERK1/2 signaling pathways. To further validate the correlation, CCK8 was used to detect cell viability after treatment with DEX-018 plus Akt inhibitor (MK2206) and PI3K inhibitor (LY294002). Compared with DEX-018 alone, MK2206 or LY294002 significantly decreased cell viability of HUVECs, indicating that the protective effect of DEX-018 against t-BHP induced oxidative injury was significantly weakened. It was further verified that the antioxidant and anti-apoptotic effects of DEX-018 were partly related to the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway.

Key words  danshensu; derivative; antioxidant; apoptosis; phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway
INTRODUCTION

Cardiovascular diseases are major causes of morbidity and mortality worldwide, accounting for approximately one third of all deaths. Ischemic heart disease (IHD), as one of the most common cardiovascular diseases, is caused by an imbalance between the myocardial oxygen supply and the metabolic demand of the myocardium. Great progress has been made in the treatment of IHD in the clinic, such as thrombolytic drug therapy, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG), while the consequent ischemia/reperfusion (I/R) injury that results in irreversible myocardial damage is inevitable and seriously impacts the effects of therapeutic for IHD. The mechanisms underlying the pathogenesis of I/R injury are complex, multifactorial, and not fully understood. Several mechanisms of this type of reperfusion injury, such as oxidative stress, intracellular Ca$^{2+}$ overload, mitochondrial dysfunction, apoptosis and inflammation-induced cardiomyocyte death, have been suggested. Among these, oxidative stress is an important mechanisms. Pharmacological cardioprotective strategies is an effective way to prevent cardiomyocytes necrosis and thereby salvaging the jeopardized myocardium. Unfortunately, the mortality and morbidity associated with I/R injury remain unacceptably high. The latest research has suggested that multipathway and/or multitarget strategies have a strong therapeutic effect in reducing ischemic death and improving ischemia-related morbidity and mortality. Thus, it is urgent to develop novel drugs with multiple pharmacologic actions to protect against I/R.

Chinese herbal medicines have been widely used in China and other Asian countries for a long time, and have attracted increasing attention for the treatment of IHD under the guidance of multitarget strategies. Danshen, the dried root of Salvia miltiorrhiza, which is one of the most popular Chinese herbs, has been widely used for the treatment of IHD and other cardiovascular disorders. Danshensu (DSS) is one of the most widely used water-soluble active ingredients of Danshen. Numerous studies have shown that DSS can improve microcirculation, inhibit platelet adhesion and aggregation, and it has antioxidant, anti-inflammatory and other pharmacological activities. Unfortunately, the further development and clinical application of DSS have been impeded by its poor bioavailability,
which may be due to its high hydrophilicity, poor absorption, and low pharmacological potency.\textsuperscript{13} Therefore, to overcome its flaws and enhance its therapeutic efficacy, the structure of DSS needs to be modified. Recently, many DSS derivatives have been designed and synthesized to yield better drug candidates with enhanced activities.\textsuperscript{14}

Increasing evidence indicates that oxidative stress is the major pathological process leading to myocardial injury after reperfusion. Hence, inhibiting oxidative damage would be a crucial therapeutic strategy for treating I/R injury. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, EDA) is a free radical scavenger that has been clinically used for the treatment of ischemic stroke.\textsuperscript{15} It has been identified as a potential protective agent for cardiovascular diseases resulting from inflammation, oxidative stress and/or cytokine-induced apoptosis.\textsuperscript{16}

Therefore, we designed the compound DEX-018 (Fig. 1), which consists of the DSS and edaravone pharmacophore. We esterified the carboxy of DSS with EDA to obtain compound DEX-018 and expected the new compound to be hydrolyzed by esterase to yield DSS and EDA. DSS and EDA were generated after hydrolysis, and DSS not only retained its pharmacological activity, but also exhibited a stronger antioxidant effect; thus a synergistic protective effect was achieved. The fat solubility of DSS was also enhanced. The pharmacodynamics of DEX-018 against tert-butyl hydrogen peroxide (t-BHP) induced oxidative damage in human umbilical vein endothelial cells (HUVECs) was studied in vitro, to investigate the cardioprotective effects and possible mechanisms of DEX-018.

MATERIALS AND METHODS

Chemistry The synthesis of the final product DEX-018 was carried out following the synthesis procedure shown in Chart 1. In brief, the esterification reaction of sodium danshensu (DSSNa) with anhydrous methanol in the presence of a catalytic amount of concentrated sulfuric acid gave intermediate 1, which was then reacted with benzyl bromide and potassium carbonate in DMF to give intermediate 2. The methyl ester of 2 was hydrolyzed with NaOH in tetrahydrofuran to give the desired carboxylic acid intermediate 3. The hydroxy group of 3 was esterified with acetic anhydride in dichloromethane to yield intermediate 4. The carboxylic group of 4 was converted to the corresponding acid chloride.
by reacting it with oxalyl chloride. The corresponding acid chloride was unstable and reacted immediately with edaravone to afford intermediate 5. The removal of the benzyl protecting group of intermediate 5 by catalytic hydrogenolysis over 10% Pd/C gave the final product DEX-018. The corresponding data for the synthetic intermediates and final compound can be found in the Supplementary Materials.

Reagents and antibodies  Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Bank of Shanghai Branch of Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) was obtained from Gibco Life Technologies Co. (Australia). Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered solution (PBS) were purchased from HyClone (Thermo Fisher). HUVECs were cultured in DMEM supplemented with 10% FBS and 1% penicillin streptomycin, at 37°C with 5% CO₂ in a humidified incubator. Cells in the exponential phase were collected for subsequent experiments. Tert-butyl hydroperoxide (t-BHP) was purchased from Sigma-Aldrich (Beverly, MA, USA). Antibodies against total- and p-ERK1/2, total- and p-Akt, caspase-3, Bcl-2 and Bax were purchased from Cell Signaling Technology (Danvers, MA). β-actin antibody was obtained from Abcam (Cambridge, MA, USA).

Cell viability assay  The effect of the synthesized conjugate on the viability of t-BHP treated HUVECs was investigated by Cell Counting Kit-8 (CCK8) analysis supplied by the manufacturer (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 4×10⁴ cells per well and allowed to attach overnight in DMEM containing 10% FBS. Then, the HUVECs were pretreated with DSS (50 μM), EDA (200 μM) or different concentrations of DEX-018 for 24 h before exposure to t-BHP (150 μM) for 2 h. Then, 100 μL of CCK-8 solution was added to each well and incubated at 37°C for 1h. The cells were pretreated with LY294002 (a PI3K inhibitor, 2 μM) and MK2206 (an Akt inhibitor, 2 μM) for 1 h, and then incubated with DEX-018 (50 μM, 100 μM) for 24 h before stimulated by t-BHP (150 μM) for 2 h. The absorbance was measured at 450 nm (Bio-Rad Instruments, USA).

Adhesion assay  A total of 5×10⁵ HUVECs per well were allowed to adhere in 6-well plates overnight. And then, pretreated with DSS (50 μM) and different concentrations of DEX-018 (5 μM, 50 μM, 100 μM) for 24 h before exposure to t-BHP (150 μM) for 2.5 h.
cell suspension of each sample was transferred to a 96-well plate. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed twice with PBS, and then the residual adherent cells were stained with Hoechst 33342 for 15 min. The number of adherent cells was counted at a fluorescence microscope (100× magnification).

Lactate dehydrogenase (LDH) release assay  HUVECs in 6-well plates were pretreated with DSS, EDA and DEX-018 for 24 h and then stimulated with t-BHP (150 μM) for 2.5 h. The cell culture medium supernatant of each sample was transferred to a 96-well plate, and LDH release was detected using an LDH cytotoxicity detection kit (Beyotime Biotech, China) according to the manufacturer’s instructions. The absorbance value at 490 nm was measured with a microplate reader.

Measurement of reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD)  A total of 3×10^5 HUVECs per well were incubated in 24-well plates overnight. The HUVECs were washed twice with PBS. Then, the cells were stained with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (10 μM) at 37°C for 20 min after being stimulated with t-BHP (150 μM) for 2.5 h. ROS generation in HUVECs was detected by confocal microscopy.

To further elucidate the antioxidant activity of DEX-018 on t-BHP induced oxidative injury, malondialdehyde (MDA) production and intracellular superoxide dismutase (SOD) were quantified after t-BHP incubation. HUVECs in 6-well plates were pretreated with DSS, EDA and DEX-018 for 24 h, then stimulated with t-BHP (150 μM) for 2.5 h. The MDA level was measured with an MDA assay kit (Beyotime Biotech, China), and SOD activity was assessed using hydroxylamine method with a total SOD assay kit.

Analysis of t-BHP induced apoptosis of HUVECs  To assess the anti-apoptotic activity of DEX-018, Hoechst staining was performed to observe the morphological changes in cells treated with the indicated concentrations of DEX-018 compared with t-BHP treated cells. A total of 7×10^4 HUVECs per well were allowed to adhere to 24-well plates overnight. Then, the cells were pretreated with DSS and different concentrations of DEX-018 for 24 h before exposure to t-BHP (150 μM) for 2.5 h. Subsequently, after washing 2 times with PBS, the cells were stained with Hoechst 33342 for 15 min. The morphological changes were observed.
by fluorescence microscopy.

**Western blot assay** For whole cell extraction, cells were washed twice with ice-cold PBS and lysed in RIPA buffer with protease & phosphatase inhibitor. After centrifugation (4 °C, 15 min, 12,000 x g), the supernatant was collected and the samples were prepared for Western blot analysis. Total proteins were extracted from HUVECs and the protein levels were measured by using the Bradford assay (Beyotime Biotech, China). The proteins were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies including p-Akt (1:1000), Akt (1:1000), Bcl-2 (1:1000), Bax (1:1000), p-ERK1/2 (1:1000, Abcam), anti-ERK1/2 (1:1000, Abcam), caspase-3 (1:1000), and β-actin (1:1000) at 4°C overnight. The membranes were then washed in TBST and incubated with appropriate horseradish peroxidase (HRP) -conjugated secondary antibodies in the dark for 1 h at room temperature. The blots were visualized with an Odyssey Fc. detection system (LI-COR) and the band intensity was analyzed by scanning densitometry with a Tanon Image System (Tanon, China).

**Statistical analysis** The data were obtained from at least three independent experiments and are shown as the means ± SEMs. Statistical analysis was performed using GraphPad Prism 5.0 software with one-way ANOVA or t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**Protective effects of DEX-018 on t-BHP- injured HUVECs** First, the protective effect of different concentrations of DSS on the oxidative damage induced by t-BHP was investigated. As shown in Fig. 2A, DSS significantly inhibited the decline in cell viability induced by t-BHP, and 50 μM DSS had the best protective effect; therefore, so we chose 50 μM DSS as the positive control for subsequent experiments.

The protective effects of DEX-018 on the viability of t-BHP treated HUVECs were investigated by CCK8 analysis. DSS served as a positive control in the experiments. To evaluate the optimal concentration for the following study, HUVECs were incubated with
different concentrations of DEX-018 (5 μM, 50 μM, 100 μM, 200 μM) before t-BHP induced for 2 h. As shown in Fig. 2B, t-BHP (150 μM) treatment decreased cell viability to approximately 60%, but pretreatment for 24 h with different concentrations of DEX-018 significantly attenuated t-BHP induced oxidative injury and improved cell viability. DEX-018 was observed to have a better protective effect on HUVECs than DSS at the same concentration. Thus, 100 μM DEX-018 was used for subsequent experiments.

$t$-BHP leads to LDH release, which is often used as an indicator of cytotoxicity. To further confirm the protective effect of DEX-018 against $t$-BHP induced cell oxidative damage, LDH leakage was measured in HUVECs pretreated with DEX-018. LDH release was observed after stimulation with $t$-BHP (150 μM) for 2 h. The loss of cell viability induced by $t$-BHP was accompanied by a significant increase in LDH release, reflecting that $t$-BHP reduced the membrane integrity in HUVECs. In contrast, pretreatment with DEX-018 (50 μM, 100 μM) and DSS decreased LDH release, and DEX-018 (100 μM) exhibited higher activity than DSS and EDA (Fig. 2C).

**DEX-018 improved adhesion function in t-BHP induced HUVECs**  The effects of DEX-018 on adhesion function were observed by performing a cell adhesion test. As shown in Fig. 3, the number of adherent cells was significantly reduced after incubation with 150 μM $t$-BHP for 3 h. Pretreatment with 50 μM or 100 μM DEX-018 greatly increased the number of adherent cells compared with that in the model group. The results indicated that DEX-018 significantly improved the adhesion of HUVECs treated with by $t$-BHP.

**Antioxidant activity of DEX-018 on t-BHP-injured HUVECs**  To demonstrate the protective effect of DEX-018 on $t$-BHP induced oxidative damage in HUVECs, SOD and MDA were measured. SOD activity and MDA production reflect the ability to scavenge ROS and prevent lipid peroxidation. MDA, which participates in the onset of atherosclerosis and has mutagenic and cytotoxic effects, is widely used for the assessment of lipid peroxidation. DEX-018 significantly increased SOD activity and decreased MDA level compared with those in the model group, and DEX-018 was as effective or more effective than DSS and EDA (Fig. 4A, B). These results demonstrated the antioxidant properties of the compound, which might be one of the mechanisms underlying its cardioprotective effect.
It has been reported that reactive oxygen species (ROS) are related to a variety of cardiovascular diseases and appears to be an early and critical event in oxidant-induced cytotoxicity.\textsuperscript{20} We further used DCFH-DA, a ROS fluorescence probe emitting green fluorescence upon oxidation by ROS, to measure the intracellular ROS level. As shown in Fig. 4C, only weak fluorescence was observed in the control group compared with the \textit{t}-BHP group. DEX-018 reduced \textit{t}-BHP induced intracellular ROS generation significantly in a concentration-dependent manner, and DEX-018 at the concentration of 100 µM decreased ROS almost to the control level. In addition, the antioxidant effect of DEX-018 (100 µM) was better than that of DSS.

\textit{DEX-018 attenuated \textit{t}-BHP induced apoptosis in HUVECs} To measure the anti-apoptotic activity of DEX-018, Hoechst 33342 staining was performed to observe the nuclear morphological changes induced by \textit{t}-BHP. As shown in Fig. 5A, typical apoptotic features, such as nuclei-shrunk and nuclear condensation, which are indicated by the red arrows, were significantly exhibited in the \textit{t}-BHP group. Compared with the \textit{t}-BHP group, the administration of DEX-018 (5 µM, 50 µM, 100 µM) and DSS resulted in a concentration-dependent decrease in cell apoptosis. Meanwhile, apoptosis-related proteins, such as Bcl-2, Bax and caspase-3, were analyzed by Western blotting. caspase-3 is considered to be the most important executioner caspases and is activated by the initiator caspases.\textsuperscript{21} In addition, Bcl-2 family members are critical for the regulation of apoptosis, and Bcl-2 and Bax are the most important regulatory genes in the Bcl-2 family for determining cells survival or apoptosis.\textsuperscript{22, 23} As shown in Fig. 5B, the expression of Bcl-2 and the Bcl-2/Bax ratio induced by \textit{t}-BHP were significantly decreased compared with those in the control group, and the expression of Bax and caspase-3 was increased. Meanwhile, like DSS, DEX-018 pretreatment effectively reversed this phenomenon.

\textit{PI3K/Akt activation was involved in the protective effect of DEX-018 on \textit{t}-BHP induced HUVECs injured} To further investigate the possible underlying mechanisms, the involvement of Akt and ERK1/2 was detected by Western blot analysis. As shown in Fig. 6A, and Fig. 6B, pretreatment with DEX-018 significantly increased the expression of p-Akt and p-ERK1/2 compared with that in the \textit{t}-BHP group. The PI3K/Akt signaling cascades play...
important roles in both the apoptosis and survival of cardiomyocytes, and the activation of the PI3K/Akt signaling pathway inhibits apoptosis and protects the heart and brain from ischemia/reperfusion injury. To confirm that the anti-apoptotic effect of DEX-018, we examined the effects of the coadministration of DEX-018 and LY294002 (a PI3K inhibitor, 2 μM) or MK2206 (an Akt inhibitor, 2 μM) on t-BHP induced apoptosis. As shown in Fig. 6C, pretreatment with LY294002 or MK2206 significantly reversed the beneficial effect of DEX-018 on t-BHP induced cell death. The results showed that PI3K and Akt inhibitors significantly attenuated the protective effects of DEX-018, demonstrating that the inhibition of cell apoptosis with DEX-018 was dependent on PI3K/Akt pathway activation to some extent.

DISCUSSION

Danshensu, has extensive pharmacological effects and potential clinical application value. However, the instability of the phenolic hydroxyl leads to poor liposolubility and low bioavailability; this, it cannot easily pass through the blood-brain barrier. Previous reports have suggested that edaravone suppresses oxidative stress and protects the heart against septic myocardial injury and dysfunction by activating the HIF-1α/HO-1 pathway. In the present study, we introduced edaravone, a powerful free radical scavenger, into the structure of DSS based on multitarget strategy. The new derivative DEX-018 was synthesized by esterification of the carboxy group of DSS and the hydroxyl group of edaravone. DEX-018 not only increased the stability and liposolubility of these compounds, but also exhibited an increased antioxidant effect, allowing it to achieve synergistic cardioprotective effects.

Vascular endothelial injury is considered to be the initial event of the development and progression of cardiovascular diseases (CVDs). Endothelial cells are very sensitive to oxidative stress in the development of CVDs and impaired endothelial function partly results from increased oxidative stress. Endothelial cell apoptosis is closely associated with many diseases such as coronary arteriosclerotic cardiopathy, hypertension and diabetes. HUVECs are commonly considered a tool in the study for the mechanisms involved in the pathogenesis of cardiovascular diseases. In this study, we established a stable t-BHP induced injury
model to investigate the protective effect and the possible mechanism of DEX-018 in HUVECs.

In this study, the results showed that DEX-018 significantly increased cell viability and reduced LDH leakage in t-BHP induced oxidative damage in HUVECs and had a similar or even better effect than DSS in increasing cellular activity. DEX-018 was proven to have a marked cellular protective effect and anti-apoptotic activity by regulating the expression of the apoptosis-associated proteins Bcl-2/Bax and caspase-3. Based on the obtained results, the novel compound protected HUVECs against apoptosis induced by t-BHP. It is well-known that the members of the Bcl-2 family play important roles in mitochondria-dependent activation of caspase cascades, which are composed of a series of anti- or pro-apoptotic regulators. Bcl-2 protein is known to promote cell survival and suppress cell death, whereas Bax is a pro-apoptotic protein that promotes or accelerates cell death. Our data demonstrated that DEX-018 markedly increased the level of Bcl-2 and decreased the Bax/Bcl-2 ratio and the level of the apoptosis-associated protein caspase-3 compared with those in the in t-BHP group. In addition, vascular endothelial cell adhesion and injury are important in the initial period of IHD, and it was found that the adhesion of HUVECs induced by t-BHP was significantly impaired by pretreatment with DEX-018.

It is well known that ROS are related to various cardiovascular diseases and that ROS production appears to be an early and critical event that leads to myocardial I/R injury in oxidant-induced cytotoxicity. SOD activity and MDA production reflect the capability to scavenge ROS and prevent lipid peroxidation. The results suggested that DEX-018 showed potential antioxidant activity, which was characterized by increased SOD activity and decreased MDA levels in t-BHP induced HUVECs. However, there was no significant difference in SOD activity or MDA production between cells treated with DEX-018 and those treated with DSS. Whereas, there may be another way for the novel compound to act as an antioxidant agent. Therefore, the mechanism of the antioxidant activity of DEX-018 needs further investigation. Ischemia/reperfusion injury occurs when blood and oxygen supply to the ischemic heart is restored, leading to an "explosion" of ROS in mitochondria, which promotes the aggravation of ischemic tissue injury. Therefore, it is of great significance to
detect intracellular ROS levels. We further investigated intracellular ROS levels. Our results indicated that pretreatment with DEX-018 significantly reduced t-BHP induced intracellular ROS production in a concentration-dependent manner. In addition, the effect of DEX-018 was better than those of DSS. The above experiments indicated that these compounds exerted potent protective effects against oxidative damage.

The PI3K/Akt pathway is one of the well-documented pathways involved in protection against oxidative stress and plays a critical role in promoting cell survival in the heart.32) Akt regulates cell survival by phosphorylating different substrates to directly or indirectly regulate apoptosis. The ERK1/2 pathway is one of the three major MAPK signaling pathways. The activation ERK1/2 is related to myocardial apoptosis, and the inhibition of ERK1/2 is beneficial for cardiac functional recovery.33) In the present study, we found that compared with t-BHP group, DEX-018 group exhibited significantly increased protein phosphorylation of Akt and decreased protein phosphorylation of ERK1/2, suggesting that the protective effect of DEX-018 was at least partly due to its ability to regulate the PI3K/Akt and ERK1/2 signaling pathways. Our results clearly demonstrated that DEX-018 plus LY294002 (PI3K inhibitor) group and DEX-018 plus MK2206 (Akt inhibitor) group significantly diminished the protective role of DEX-018, further confirming the role of the PI3K/Akt pathway in mediating the protective effect of DEX-018 against t-BHP induced oxidative injury in HUVECs.

Taken together, the results showed that DEX-018 protected HUVECs against t-BHP-induced oxidative injury by regulating the PI3K/Akt and ERK1/2 signaling pathways, which suggested that the novel danshensu derivative DEX-018 can protect against vascular endothelial cell injury and may reduce the incidence of cardiovascular disease. Further study is in progress and the novel compound merits further investigation as a potential cardiovascular protective candidate.

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Fig. 1. Design strategies for danshensu derivative DEX-018.
Chart 1. Synthesis of the DEX-018
Fig. 2. Protective effects of DEX-018 on injured HUVECs induced by t-BHP. Cells were pretreated with different concentrations of DSS and DEX-018 for 24 h and then stimulated with or without t-BHP (150 μM) for 2 h. (A) Effects of different concentrations of DSS on cell viability induced by t-BHP in HUVECs. (B) Effects of different concentrations of DEX-018 on cell viability induced by t-BHP in HUVECs. (C) Effects of DEX-018 on LDH leakage. The data were represented as the means ± SEM in three independent experiments, *p < 0.05 and **p < 0.01 vs t-BHP group. ***p < 0.01 vs control group.
Fig. 3. Effects of DEX-018 on cell adhesion induced by t-BHP in HUVECs. Cells were pretreated with DSS (50 μM) or different concentrations of DEX-018 (5, 50, 100 μM) for 24 h and then stimulated with or without t-BHP (150 μM) for 2 h.
Fig. 4 Antioxidant activity of DEX-018 on injured HUVECs induced by t-BHP. The cells were incubated with different concentrations of DSS or DEX-018 for 24 h, then stimulated with or without t-BHP (150 μM) for 2h. (A) Effects of DEX-018 on SOD activity. (B) Effects of DEX-018 on MDA level. (C) The levels of ROS in HUVECs cells after pretreated with DEX-018. The data were represented as the means ± SEM of three independent experiments, *p<0.05 or **p<0.01 vs t-BHP group; #p<0.05 vs control group. EDA: Edaravone.
Fig. 5. DEX-018 inhibited t-BHP induced apoptosis in HUVECs. (A) Effects of DEX-018 on apoptosis in HUVECs induced by t-BHP (150 μM). Apoptosis of HUVECs was observed with Hoechst33342 nuclear staining. Arrowheads in the pictures indicate the nuclei of apoptotic cells. Scale bar: 50 μm. (B) Effects of DEX-018 on the expression of Bcl-2, Bax and caspase-3 were measured by Western blot. Densitometric data were expressed as the means ± SEM of three independent experiments; *p < 0.05 vs control group, and *p < 0.05 vs t-BHP group.
Fig. 6. PI3K/Akt signaling pathway was involved in the DEX-018 treated HUVECs induced with t-BHP. (A) (B) p-Akt, Akt, ERK1/2, p-ERK1/2 were measured by Western blot. (C) Effects of MK2206 (2 µM) and LY294002 (2 µM) on cell viability induced by t-BHP in HUVECs. The data were represented as the means ± SEM of three independent experiments, #p<0.05 and ##p<0.01 vs control group; *p<0.05 and **p<0.01 vs t-BHP group; &p<0.05 vs DEX-018 treated group; MK: MK2206 (Akt inhibitor), LY:LY294002 (PI3K inhibitor).