Cardioprotection of a Novel Danshensu Derivate Against Oxidative Injury and Mitochondrial Dysfunction in Vitro and Myocardial Ischemia/Reperfusion Injury in Rat

ABSTRACT

Objectives Myocardial ischemia/reperfusion injury remains an important clinical problem in the world. Extensive research have focused on strategies for attenuating myocardial ischemia and reperfusion injury. Danshensu (DSS) and tetramethylpyrazine (TMP) are important cardioprotective components isolated from the traditional Chinese herbs Danshen and Shuanchuang, respectively. D2T is a novel conjugate with two molecules of DSS introduced to a TMP through an ester bond. This study aims to explore the cardioprotection of D2T and its mechanisms of action.

Methods and Results H9c2 cardiomyocytes were treated with tert-butyl hydroperoxide (t-BHP) to induce oxidative stress in vitro. D2T pretreatment protected cardiomyocytes from t-BHP-induced oxidative injury and mitochondrial dysfunction. Western blot analysis showed that D2T activated phosphorylation of PI3K and Akt and induced HO-1 protein expression together with its upstream regulator Nrf2. Wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor, partially abolished the restoration of cell viability offered by D2T. In a rat myocardial ischemia and reperfusion model, intravenous D2T significantly reduced infarct size.

Conclusions The cardioprotection of D2T is mediated by inhibiting oxidative injury and mitochondrial dysfunction. D2T acts by the activation of PI3k/Akt and Nrf2/HO-1 signaling pathways, suggesting D2T may be an effective treatment for myocardial ischemia/reperfusion injury.

KEYWORDS danshensu derivate, myocardial ischemia/reperfusion, oxidative stress, mitochondrial dysfunction, PI3K/Akt pathway

INTRODUCTION

Ischemia/reperfusion (I/R) injury remains a major cause of morbidity and mortality after cardiac surgery and myocardial infarction1-3. Prompt revascularization and restoration of blood flow are still the most effective therapeutic approach to ischemia. However, reperfusion necessary to recover oxygen and nutrient supplies exacerbates cardiac ischemic injury and this irreversible and additional damage is called reperfusion injury.

Oxidative stress arises from the disruption of the balance between oxidant and antioxidant1. Increasing evidence have suggested that oxidative stress plays a pivotal role in the pathogenesis of myocardial ischemia/reperfusion injury4. Intracellular free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key players in oxidative stress. The excess ROS/RNS generated upon ischemia and reperfusion leads to cell/tissue injury including lipid peroxidation, protein modification, and DNA damage4. Recently, numerous studies have demonstrated the protective effects of antioxidants modulating ROS generation against myocardial ischemia/reperfusion injury5-8.

Mitochondrial dysfunction is another major player in the progression of myocardial ischemia/reperfusion injury, and is also inter-related with oxidative stress.

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stress\(^9\). The blockage of oxidative phosphorylation upon ischemia/reperfusion initiates mitochondrial dysfunction, leading to mitochondrial ROS overproduction, mitochondrial membrane potential depletion, adenine nucleotide levels alteration and mitochondrial permeability transition pore (mPTP) opening and ultimately cell death\(^{10}\). Therefore, mitigation of mitochondrial dysfunction is a promising strategy for cardioprotection\(^9,11\).

Danshen (Salvia miltiorrhiza) and Chuanxiong (Ligusticum Wallichii Franch) are two widely used traditional Chinese herbs for the treatment of cardiovascular and cerebrovascular diseases\(^{12–13}\). Danshensu (DSS) [(R)-3-(3, 4-dihydroxyphenyl)-2-hydroxy-propanoic acid] and tetramethylpyrazine (TMP) are active compounds isolated from Danshen and Chuanxiong, respectively. Previous studies found that both DSS and TMP could protect heart against ischemia/reperfusion injury through attenuating oxidative stress, Ca\(^{2+}\) overload and apoptosis\(^{14–17}\). Co-administration of DSS and TMP have been reported to significantly reduce myocardial infarct size and restore normal cardiac function, showing synergistic or additive effect on cardiovascular system\(^{18–20}\). However, their therapeutic efficacy was limited due to weak stability or pharmacokinetic properties. In order to enhance their synergistic effect, we introduced two molecules of DSS to a TMP through ester bond to synthesize a novel compound named D2T (Fig. 1). We herein mainly reported the cardioprotective effects of D2T against t-BHP induced oxidative stress and mitochondrial dysfunction as well as its mechanisms of action.

MATERIALS AND METHODS

D2T was synthesized in our laboratory (>98%, purity). DSS and TMP were purchased from Honsan Biotechnology and Banghai Chemical Company, respectively. Salvianolate (SAL) injection was purchased from Shanghai Green Valley Pharmaceutical Co., Ltd. Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco. 2,3,5-triphenyl-tetrazolium chloride (TTC) were purchased from Sigma-Aldrich unless otherwise indicated. D2T was synthesized in our laboratory (>98%, purity) and used for the experiment. All other reagents were purchased from Sigma-Aldrich.

Cell culture and treatment

H9c2 cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified incubator (Thermo Scientific) containing 5% CO\(_2\). When cells reached 70–80% confluence, D2T was added to the cells and incubated for 2 h before t-BHP (150 μM) exposure.

Animal care

Male Sprague-Dawley (SD) rats were obtained from the Experimental Animal Center of Guangzhou University of Chinese Medicine, weighing between 220 and 250 g. The animals were housed under 12-h light-dark cycle and at a controlled temperature of 23 ± 2°C, with free access to food and water. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Jinan University.

Cell viability assay

Cell viability was evaluated by the MTT assay. Briefly, H9c2 cells were seeded in 96-well plates at a density of 1 × 10\(^4\) cells per well and were cultured for 24 h. After treatment with D2T (10, 30, 100, 300 μM) for 2 h, t-BHP (150 μM) was added. The cells were cultured for another 4 h. MTT was then added to each well, and the cells were cultured for 4 h at 37°C. Afterwards, DMSO was added to dissolve the formazan crystals, and the absorbance at 570 nm was measured by a microplate reader (Bioteks).

Apoptosis and necrosis assay

Cell apoptosis and necrosis were assessed by the Hoechst 33342 and PI double staining. Following D2T and t-BHP treatment, Hoechst 33342 (5 μg/ml) and PI (1 μg/ml) were added. The cells were incubated for 30 min at 37°C. Cell images were observed under a fluorescence microscope (Olympus) using excitation/emission of 340/460 nm and 535/615 nm, respectively, and were analyzed by the Image J Software.

Measurement of LDH release and MDA content

H9c2 cells (1 × 10\(^4\) cells/ml) were seeded in 6-well plates. After 24 h of culture, D2T was added and the cells were incubated for 2 h. Then, t-BHP (150 μM) was added to the medium, and the cells were cultured for another 4 h. At the end of the incubation, LDH release and MDA content were assessed with the corresponding detection kits.

Measurement of ROS, O\(_2^-\) and ONOO\(^-\)

Cellular levels of total ROS, O\(_2^-\) and ONOO\(^-\) were, respectively, detected using DCFH-DA, DHE and DHR 123. Briefly, after pretreatment with D2T, the H9c2 cells were incubated with t-BHP (150 μM) for 40 min. The cells were washed with fresh DMEM and incubated with DCFH-DA (10 μM), DHE (10 μM) or DHR 123 (10 μM), respectively, in the dark at 37°C for 30 min. Cellular fluorescence was recorded with a fluorescent microscope.
microscope and was quantified through a microplate reader (Biotek) at excitation/emission of 480/530 nm, 535/610 nm and 505/529 nm, respectively.

**Determination of mitochondrial membrane potential (ΔΨ)**

Changes in ΔΨ were assessed with the cationic reagent JC-1. Briefly, H9c2 cells were incubated with JC-1 (10 μg/ml) for 30 min at 37°C. The changes in fluorescence were measured with a fluorescence microscope (Olympus). For quantification, JC-1 fluorescence intensity of monomer and aggregates was measured using a fluorescence microplate reader at excitation/emission of 488/525 nm and 535/590 nm, respectively. The ΔΨ was calculated as the JC-1 red/green fluorescence intensity ratio.

**Mitochondrial isolation and mitochondrial swelling**

Mitochondria were isolated using the tissue mitochondria isolated kit according to the manufacturer’s instructions. Freshly isolated mitochondria (1 mg/ml) were incubated in respiratory buffer (125 mM sucrose, 50 mM KCl, 1 mM MgCl₂, 2 mM KH₂PO₄, 5 mM HEPES, 5 mM succinate, 2 μM rotenone, pH = 7.2) at 37°C. After pretreatment with D2T for 20 min, 100 μM CaCl₂ was added and the absorbance at 540 nm was immediately recorded for a period of 40 min. Mitochondrial swelling was assessed as a decrease in light absorbance at 540 nm immediately after stimulation with 100 μM CaCl₂.

**ATP content detection**

ATP content was determined using an ATP assay kit. Briefly, cells were lysed and centrifuged at 10,000 × g for 10 min. The supernatants (100 μl) were then mixed with reaction buffer (100 μl) in a 96-well plate, and the luminescence (RLU) was measured by a microplate reader. Cellular ATP levels were expressed as nmol/mg proteins.

**Western blotting assay**

H9c2 cells were lysed in an ice-cold RIPA buffer (Beyotime). After quantitation of protein concentration with the BCA protein assay kit (Beyotime), a total of 30 μg cell lysate was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then was transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fatted milk for 2 hours at room temperature, and was subsequently incubated overnight at 4°C with primary antibodies. The species-appropriate secondary antibody conjugated with horseradish peroxidase was then incubated for another 2 h at room temperature. Proteins were detected by an advanced enhanced chemiluminescence (ECL) system. Band density was quantified using the Carestream Molecular Imaging software (CarestreamHeltah, Inc).

**Cardioprotection of a novel danshensu derivate**

Male SD rats weighing 220–250 g were randomized into six groups as follows: (i) Sham group; (ii) I/R group; (iii) I/R + D2T (10 mg/kg) group; (iv) I/R + D2T (30 mg/kg) group; (v) I/R + D2T (60 mg/kg) group; (vi) I/R + SAL (10 mg/kg) group. 6–8 animals were assigned to each group. Rats were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection) and were ventilated with a small animal respirator Thoracotomy was performed, and the left anterior descending (LAD) coronary artery was ligated 2–3 mm from its origin between the pulmonary comusand left atrium with a 6–0 silk suture. After 30 min of LAD ligation, the ligature was removed to allow reperfusion. Compounds were administrated twice by intravenous injection. The first administration was given at 15 min after occlusion and the second at 3 h following reperfusion. The sham group was subjected to the same operations except ligation. After 24 h of LAD ligation, the hearts were quickly excised and frozen, and then sliced into 2 mm thick sections. The slices were stained with 2% TTC at 37°C for 15 min and then fixed for 24 h in 4% paraformaldehyde solution. The image was then captured and analyzed by Image J software. The infarct size was expressed as the ratio of infarct area to that of the whole left ventricular area.

**Statistical analysis**

All results were presented as mean ± SD. The statistical analysis of results was performed using the GraphPad Prism software (version 5.0). Statistical differences between groups were analyzed by one-way analysis of variance or Student’s t-test when appropriate. P value less than 0.05 was regarded as statistically significant.

**RESULTS**

**Protective effects of D2T against t-BHP-induced cardiomyocyte injury**

The cytotoxicity of D2T in H9c2 cells was first examined using the MTT assay. As shown in Fig. 2A, D2T up to 300 μM did not display any cytotoxicity after 12-h incubation with the cells. t-BHP (150 μM) caused approximately 50% loss of cellular viability, which was concentration-dependently attenuated by pretreatment with D2T (10, 30, 100, 300 μM) (Fig. 2B). Salvianolate injection (SAL) was used as a positive control in the study. D2T was far more effective than SAL and the mixture of DSS and TMP. Results in Figure 2C and D demonstrated that D2T significantly decreased t-BHP-induced LDH release and MDA formation.

**D2T inhibited t-BHP induced H9c2 cell apoptosis and necrosis**

Hoechst 33342 and PI staining were employed to assess the effects of D2T on t-BHP-induced apoptosis.
and necrosis. Apoptotic cells with cell shrinkage and nuclear condensation were indicated by red arrows, and necrotic cells were characterized by PI-positive staining (Fig. 3A). As shown in Fig. 3B, the number of apoptotic and necrotic cells was significantly higher in t-BHP-injured cells, and this increase was dramatically prevented by D2T treatment.

**D2T reduced the t-BHP-induced reactive free radicals overgeneration**

To investigate whether D2T can reduce the intracellular reactive free radicals in H9c2 cells, DCFH-DA, DHE and DHR 123 fluorescence were employed. The images (Fig. 4A) and quantified results (Fig. 4B–D) indicated that t-BHP injury remarkably increased levels of total ROS and O$_2^-$ and ONOO$^-$. The increases were significantly decreased by D2T in a concentration-dependent manner.

**D2T attenuated t-BHP induced mitochondrial dysfunction**

To examine the effects of D2T on t-BHP-injured mitochondrial activities, mitochondrial transmembrane potential (ΔΨ), the intracellular ATP level, and mitochondrial swelling were determined. As shown in Figure 5A, exposure of t-BHP to cells caused a decrease in red fluorescence intensity, indicating ΔΨ dissipation. D2T treatment from 10 to 100 μM significantly attenuated ΔΨ dissipation in a concentration-dependent manner (Fig. 5B). The results in Figure 5C showed that the intracellular level of ATP was significantly lowered after exposure to 150 μM t-BHP. D2T concentration-dependently attenuated ATP depletion induced by t-BHP in H9c2 cells. As shown in Figure 5D and E, mitochondria treated with 100 μM Ca$^{2+}$ caused a decrease in light absorbance at 540 nm, suggesting mitochondrial swelling. However, the excessive swelling was diminished by 100 μM D2T treatment. Consistent with previous results, cyclosporin A (CsA), an mPTP inhibitor, significantly blocked Ca$^{2+}$-induced mitochondrial swelling. These results suggested that D2T treatment prevented t-BHP induced mitochondrial dysfunction.

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Fig. 1 Chemical structures of DSS, TMP and D2T.

Fig. 2 D2T protected cardiomyocytes against t-BHP-induced oxidative injury. (A) H9c2 cells were treated with D2T (10, 30, 100, 300 μM) for 12 h to test the drug cytotoxicity. (B) Pretreatment with D2T (10, 30, 100, 300 μM) significantly attenuated t-BHP-induced cell damage. Effect of D2T on (C) LDH release and (D) MDA content in t-BHP treated cells. Data are presented as mean ± SD (n = 4 per group). ###P < 0.01 vs control group; *P < 0.5, **P < 0.1, ***P < 0.01 vs model group, NS, non-significant (P > 0.05).
**Fig. 3** D2T inhibited t-BHP-induced cardiomyocyte apoptosis and necrosis. (A) Fluorescence images of morphological apoptosis indicated by red arrows. (B) Quantitative percentages of apoptotic (Hoechst positive) and necrotic (PI positive) cells. Data are presented as mean ± SD. (n = 4 per group). **P < 0.01 vs control group in both apoptotic and necrotic cells; ***P < 0.001 vs model group in necrotic cells; ΔP < 0.5, ΔΔΔP < 0.001 vs model group in apoptotic cells.
Fig. 4 D2T alleviated t-BHP-induced intracellular free radical generation in cardiomyocytes. (A) Fluorescence images of DCFH-DA, DHE and DHR 123 fluorescence staining to detect intracellular ROS, \(O_2^-\) and ONOO\(^-\) respectively. Bar graphs showing the quantitative analysis of fluorescence intensity for (B) ROS, (C) \(O_2^-\) and (D) ONOO\(^-\) levels. Data are presented as mean ± SD (n = 4 per group). 

###P < 0.001 vs control group; **P < 0.1, ***P < 0.001 vs model group.
Fig. 5  D2T attenuated mitochondrial dysfunction. Effects of D2T on the changes of mitochondrial membrane potential (ΔΨm) (A and B), ATP levels (C) and Ca^{2+}-induced mitochondrial swelling (D and E). Data are presented as mean ± SD. (n = 4 per group). ###P < 0.001 vs control group; *P < 0.05, ###P < 0.001 vs model group.
Expression of apoptosis-related proteins

To clarify the mitochondria-mediated apoptosis involved in the protective effects of D2T against oxidative stress injury, the expression levels of mitochondrial apoptosis-related proteins were determined. Western blot analysis (Fig. 6A) demonstrated a decrease in Bcl-2 expression and an increase in Bax, cytochrome c (cyt c) and cleaved caspase-3 protein expression after t-BHP injury. D2T pretreatment caused a concentration-dependent increase in the ratio of Bcl-2/Bax (Fig. 6B) and decrease in cyt c (Fig. 6C) and cleaved caspase-3 (Fig. 6D) expression.

Effect of D2T on PI3K/Akt signaling pathway

Activation of PI3K/Akt signaling pathway has been shown to be important for cardioprotection. Western blot analysis showed that D2T could concentration-dependently increase the phosphorylation of PI3K and Akt (Fig. 7A–D). To confirm whether PI3K/Akt signaling pathway was involved in the protection of D2T against t-BHP induced cell injury, the specific PI3K inhibitor Wortmannin (Wort, 1 μM) was employed. Cells were pretreated with Wort for 30 min prior to D2T treatment. The results (Fig. 7E and 7F) showed that the D2T induced-phosphorylation of Akt with or without t-BHP injury was almost completely abolished by Wort preincubation. More importantly, pretreatment with Wort significantly attenuated the protective effects of D2T against t-BHP induced cell death (Fig. 7G), indicating that the protective effect of D2T depended on PI3K/Akt activation.

D2T activated the Nrf2/HO-1 pathway

Increasing evidence support a critical role for Nrf2/HO-1 pathway in the cellular defenses against oxidative stress. Further investigation was performed on Nrf2 and HO-1 expression after D2T pretreatment in t-BHP injured cardiomyocytes. Our result showed that t-BHP decreased the protein expression of Nrf2 and HO-1; however, D2T significantly increased Nrf2 (Fig. 8A and C) and HO-1 (Fig. 8B and D) protein expressions in a concentration-dependent manner.

D2T protected hearts against ischemia/reperfusion injury in rat

Considering the promising effect of D2T against t-BHP-induced oxidative injury in H9c2 cells, the cardioprotection of D2T was further evaluated in a rat myocardial I/R model. As shown in Figure 9, D2T administration at 10, 30 and 60 mg/kg significantly reduced the infarct size compared to that of the I/R model group. SAL was used as a positive control. The rats

Fig. 6 Effects of D2T on mitochondrial apoptosis-related protein expressions in H9c2 cells. The expression of apoptosis-related proteins was measured (A) and quantified (B, C and D) by western blot. Data are presented as mean ± SD of four independent experiments. ###P < 0.001 vs control group; *P < 0.05, ***P < 0.001 vs model group.
Fig. 7 Involvement of PI3K/Akt pathway in D2T mediated cardioprotection in H9c2 cells. The protein expression was measured by western blot. (A and B) Immunoblot and (C and D) densitometry analysis showed the protein expression of p-PI3K, p-Akt and total PI3K, Akt without wort treatment. (E) Immunoblot and (F) densitometry analysis showed the protein expression of p-Akt and total Akt with wort treatment. (G) Pretreatment with wort partially abolished the protection of D2T in t-BHP treated cells. Data are presented as mean ± SD of four independent experiments. \^{\text{##}}P < 0.1, \^{\text{###}}P < 0.01\text{ vs control group; }^*P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001\text{ vs model group; }\&\&P < 0.01, \&\&\&P < 0.001\text{ vs indicated non-wort groups.}
Fig. 8 D2T up-regulated protein expressions of Nrf2 and HO-1 in H9c2 cells. (A and B) Immunoblot and (C and D) densitometry analysis showed the effect of D2T on the protein levels of Nrf2 and HO-1. Data are presented as mean ± SD of four independent experiments. * P < 0.5, ** P < 0.01 vs control group; † P < 0.05, ‡ P < 0.01 vs model group.

Fig. 9 Cardioprotective effects of D2T against myocardial ischemia/reperfusion injury in rats. Bar graph showing the ratio of infarct area to that of the whole left ventricle (LV). Data are presented as mean ± SD. Sham group: n = 4; I/R group: n = 6; D2T group: 10 mg/kg, n = 7; 30 mg/kg, n = 6; 60 mg/kg, n = 8; SAL 10 mg/kg, n = 7. ### P < 0.001 vs sham group; † P < 0.05, ‡ P < 0.01, § P < 0.001 vs model group.

were given 10 mg/kg SAL twice a day via intravenous injection, which was equal to the clinical dosage of SAL for adult human based on the conversion formula in the rules of surface area equivalence. Our result showed that the therapeutic efficacy of D2T at 30 mg/kg was similar to that of SAL (10 mg/kg), and D2T at 60 mg/kg was superior to SAL.

DISCUSSION

In this study, we identified the cardioprotective effect of D2T on t-BHP-induced oxidative injury in H9c2 cells and acute myocardial infarction in rats. D2T conferred obvious protection on cell viability, much stronger than that of SAL and mixture of DSS and TMP in the same molarity. D2T showed priority to SAL and DSS + TMP potentially due to its water and air stability and enhanced pharmacological activity after joining two active compounds through an appropriate bond, suggesting stronger synergistic effect reached after organic conjugation between DSS and TMP.

Increasing production of ROS/RNS results in oxidative stress during ischemia/reperfusion. Hydrogen peroxide may induce self-generation of free radicals and therefore has been widely used to stimulate conditions of oxidative stress. During ischemia, ROS production is produced by mitochondrial respiratory chain complexes, and was further exaggerated by reperfusion. Primary ROS production is in the form of superoxide anion radical (O$_2^−$). Superoxide anion radical can react with nitric oxide to form peroxynitrite anion (ONOO$^−$). MDA is also used to measure the degree of membrane lipid peroxidation. In the current study, D2T pretreatment significantly decreased the generation of total ROS, O$_2^−$ and ONOO$^−$ and reduced MDA formation. These findings indicated that D2T presented radicals scavenging and antioxidant activity and therefore prevented cells from oxidative injury.

Mitochondria have become a target for therapy against cardiovascular diseases. Myocardial ischemia/reperfusion and oxidative stress cause cardiac mitochondrial dysfunction through increasing mitochondrial depolarization and swelling. Dissipation of mitochondrial membrane potential (ΔΨ) is thought to
be the early phase of mitochondrial dysfunction, which reduced ATP production and triggers the Ca\(^{2+}\)-sensitivity of mPTP, causing mitochondrial swelling, rupture of the outer membrane and ultimately cell death\(^{17,23}\). In H9c2 cells, \(t\)-BHP exposure caused mitochondrial dysfunction with \(ΔΨ\)m loss, mitochondrial swelling and ATP depletion. D2T greatly reversed these changes, indicating its mitochondria function preservation. Therefore, D2T may rescue mitochondrial from oxidative stress status via its strong ROS/RNS scavenging ability and promote cell survival largely.

Besides necrotic death, myocardial ischemia/reperfusion injury also causes apoptotic cell death. Apoptosis is a type of programmed cell death activated through two major signaling pathways, the mitochondria-mediated intrinsic pathway and death receptors-mediated extrinsic pathway\(^{24}\). The B cell lymphoma-2 (Bcl-2) superfamily of proteins, such as Bax, Bak, Bcl-2 and Bcl-XL, is central regulators of the intracellular apoptotic signaling cascades. These proteins can be subdivided into two groups, the anti-apoptotic activity (Bcl-2, Bcl-XL, Bcl-w) and pro-apoptotic activity (Bax, Bak, Bid, Bad). It has also been demonstrated that Bax is crucial to cytochrome c release from mitochondria, which are critical in mitochondria-mediated cell apoptosis\(^{25-27}\). Bcl-2 is important in cell survival and protects cardiomyocytes against various stressors. Antje Diestel et al. found that up-regulation of Bcl-2 in response to hypothermia and oxidative stress may participate in cell protection and apoptosis prevention\(^{28}\). Furthermore, Bcl-2 has been reported to protect cells against death by inhibiting activation of pro-apoptotic Bax\(^{29}\). The increased ratio of Bcl-2/Bax is believed to display anti-apoptotic effects. Our findings showed that D2T significantly reduced the number of apoptotic cells. D2T up-regulated expression of Bcl-2 and inhibited Bax protein expression, thus significantly increased the ratio of Bcl-2/Bax protein expression. D2T pretreatment also reduced \(t\)-BHP induced cytochrome c release and caspase-3 activation. These results suggested that D2T protected H9c2 cells from apoptosis through inhibition of oxidative stress-mediated mitochondrial apoptotic pathway.

The PI3K/Akt signaling has been reported to be essential for cardioprotection in response to myocardial ischemia/reperfusion injury. Akt is the key factor for exerting PI3K-dependent cell-survival responses through its downstream effectors\(^{30}\). The phosphorylation and activation of Akt could preserve mitochondrial integrity and protect cardiomyocytes against oxidative injury\(^{31-33}\). In the present study, D2T increased PI3K and Akt phosphorylation but not the total PI3K and Akt expression, which are reduced by \(t\)-BHP. Furthermore, we clearly demonstrated that Wortmannin, a specific inhibitor of PI3K, completely abolished the activation of Akt and the cardioprotection by D2T. These findings suggested that the PI3K/Akt pathway plays a crucial role in D2T’s protection against \(t\)-BHP induced oxidative injury.

Nuclear transcription factor Nrf2 is retained in the cytoplasm by its inhibitor Keap1. Ischemia and reperfusion enhances Nrf2 dissociation from Keap1, resulting in stabilization and nuclear translation of Nrf2. Nrf2 binds to antioxidant response element (ARE) and activates phase II detoxifying and antioxidant genes such as HO-1\(^{14}\), HO-1, an important antioxidant enzyme, is regarded as a sensitive and reliable indicator of cellular oxidative stress\(^{35}\). Previous studies demonstrated that the activation of Nrf2 and up-regulation of HO-1 expression conferred the protection against myocardial ischemia/reperfusion injury and oxidative stress\(^{36}\). Our data showed that D2T pretreatment significantly up-regulated Nrf2 and HO-1 protein expression in \(t\)-BHP-injured H9c2 cardiomyocytes. We thus concluded that the action of D2T protecting cardiomyocyte from oxidative injury is at least partially through Nrf2/HO-1 activation.

**CONCLUSION**

In conclusion, the novel danshensu derivate D2T effectively protected cardiomyocytes against \(t\)-BHP-induced oxidative injury by inhibiting cell apoptosis, ROS production and mitochondrial dysfunction, which may be partially attributed to the modulation of mitochondria-mediated apoptotic pathway and activation of PI3K/Akt and Nrf2/HO-1 pathways. Furthermore, D2T significantly decreased the myocardial infarct size in the rat model of myocardial ischemia/reperfusion. Therefore, D2T may be a promising new therapeutic candidate for myocardial ischemia/reperfusion injury.

**CONFLICT OF INTEREST**

There was no conflict of interest regarding this manuscript.

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**REFERENCES**


