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Isosteviol sodium protects heart embryonic H9c2 cells against oxidative stress by activating Akt/GSK-3β signaling pathway

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Received October 25, 20129, accepted November 22, 2019

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Pharmazie 75: 36-40 (2020) *doi: 10.1691/ph.2020.9851*

Oxidative stress plays a crucial role in pathogenesis of various cardiovascular diseases. Recent studies reported that isosteviol sodium (STVNa) harbor cardioprotective properties. Here, we explore the potential cardioprotective effect of STVNa on H_2O_2-induced oxidative stress on heart embryonic H9c2 cardiomyocytes and the underlying mechanism. We have found that STVNa pretreatment improved cell viability, nuclear morphology and prevented LDH release induced by oxidative stress. STVNa pretreatment also reduced production of reactive oxygen species, preserved mitochondrial function, restored biological antioxidant defense systems and prevented cell death. Western blotting analysis revealed that STVNa regulated the mitochondrial related pro- and anti-apoptotic protein (Bax and Bcl-2 respectively) levels, increased phosphorylation of Akt (ser473) and GSK-3β (ser9) and promoted binding between HK-II and mitochondria under the normal or oxidative stress conditions. LY294002, a PI3K inhibitor, abolished cytoprotective effects of STVNa by inhibiting activation of Akt and GSK-3β. Based on these findings, we conclude that STVNa protects H9c2 cells against oxidative stress by activating Akt/GSK-3β signaling pathway, which, in turn, leads to recruitment of HK-II to mitochondria and regulating Bcl2/Bax levels.

1. Introduction

Isosteviol is a stevioside derivative, obtained from the leaves of *Stevia rebaudiana* (Geuns 2003). Recently, we have developed isosteviol sodium salt (STVNa), a more soluble and injectable form of isosteviol. We have shown that STVNa reduces infarct size, restores cardiac contractility and improves hemodynamic parameters following ischaemia-reperfusion without inducing arrhythmias (Ma et al. 2007; Xu et al. 2007). At the cellular level, STVNa improved the viability of rat heart embryonic H9c2 cells when exposed to ischaemia-reperfusion and reduced intracellular reactive oxygen species (ROS) levels (Sun et al. 2018). However, the mechanism underlying cardioprotective action of STVNa is yet to be fully understood.

Oxidative stress plays a crucial role not only in the pathogenesis of myocardial ischaemia-reperfusion injury, but also in the pathogenesis of other cardiovascular diseases including heart failure and atrial fibrillation (Kurian et al. 2016). As STVNa has been recently shown to be beneficial in heart failure as well as in ischaemia-reperfusion, it was prudent to assess whether this compound protects against oxidative stress and elucidate underlying mechanism of STVNa action. Consequently, we investigated the protective effects of STVNa in oxidative stress applied on embryonic heart H9c2 cells. Here, we report that STVNa protects H9c2 cells against oxidative stress-induced mitochondria-mediated cell death through regulation of Bcl-2/Bax, Akt/GSK-3β and HK-II proteins.

Abbreviations:
STVNa: Isosteviol sodium; H_2O_2: Hydrogen peroxide; LDH: Lactated hydrogenase; ROS: Reactive oxygen species; MDA: Malondialdehyde; SOD: Superoxide dismutase; DMEM: Dulbecco's modified eagle's medium; FBS: Fetal bovine serum; DCFH-DA: 2′,7′-dichlorofluorescin diacetate; FITC: Fluoresceine isothiocyanate; BCA: Bicinchoninic acid; DAPI: 4',6-diamidino-2-phenylindole; ΔΨm: Mitochondrial membrane potential; CCK-8: Cell counting kit-8; mPTP: Mitochondrial permeability transition pore; OMM: Outer-mitochondrial membrane; IMM: Inner-mitochondrial membrane

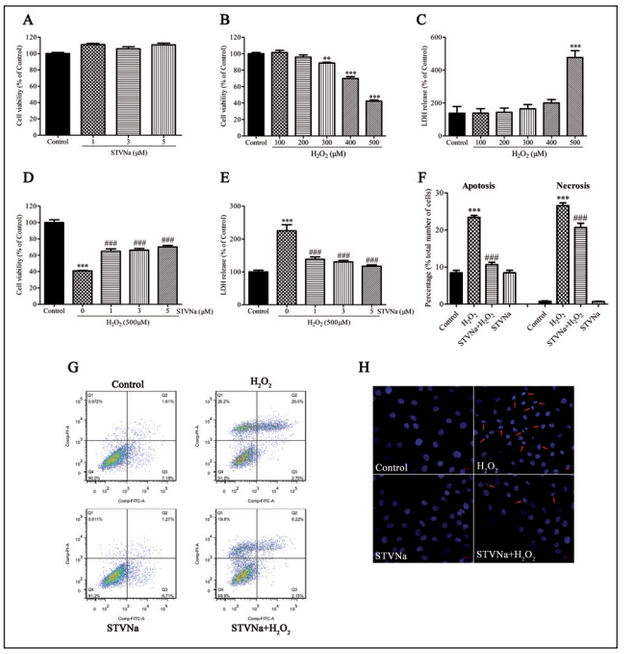

Fig. 1: Effects of STVNa pretreatment on the H9c2 cell viability. (A) Viability of cells treated with different concentration of STVNa (0-5 μM). (B-E) Viability of cells (B, D) and LDH release (C, E) from cells exposed to different concentrations of H_2O_2 (0-500 μM) under control conditions (B, C) and pretreated (D, E) with STVNa (5 μM). (F) The apoptosis ratio and necrosis ratio in cells under depicted conditions. (G) Typical images of flow cytometry. Q1 represented necrotic cells, Q2 contained non-viable apoptotic cells, Q3 contained viable apoptotic cell and Q4 contained normal cells. (H) Original images showing cell nuclei under control conditions and when exposed to 500 μM of H_2O_2 untreated and pretreated with STVNa (5 μM). In all graphs, each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the untreated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the H_2O_2 model group (n = 3-6).

2. Investigations and results

2.1. STVNa protects H9c2 cells against oxidative stress

Exposure to STVNa (1-5 μM) alone did not affect cellular viability, while exposure to H_2O_2 (100-500 μM) induced cell death, which was associated with LDH release in a concentration-dependent manner (Figs. 1A-1C). As 500 μM H_2O_2 produced death of ~50% cells, we selected that particular H_2O_2 concentration for further experimentation. STVNa (1-5 μM) protected H9c2 cells against oxidative stress induced by H_2O_2 (500 μM; Figs. 1D and 1E). Flow cytometric analysis demonstrated that apoptotic and necrotic ratio in H_2O_2 (500 μM) was significantly higher than in the control group (P<0.001; Figs. 1F and 1G), which was inhibited by 5 μM STVNa (Figs. 1F and 1G). The shape of the nuclei in control group cardiomyocytes were regular and oval. However, in H_2O_2 (500 μM) treated group, DNA fragmentation and nuclear condensation was observed (Fig. 1H). STVNa (5 μM) prevented such effect of H_2O_2 (Fig. 1H).

2.2. STVNa regulates intracellular ROS levels in H9c2 cells

H_2O_2 (500 μM) increased ROS levels in H9c2 cells (Fig. 2A and 2B), which was prevented by STVNa (5 μM; Figs. 2A and 2B). To determine antioxidant capability of STVNa during H_2O_2 induced oxidative stress, SOD activity and MDA levels were assayed using the lysates of H9c2 cardiomyocytes. As shown in Figs. 2C and 2D, exposure to H_2O_2 (500 μM) decreased SOD activity and significantly increased MDA production (Figs. 2C and 2D). STVNa (5 μM) inhibited those effects of H_2O_2 (500 μM; Figs. 2C and 2D).

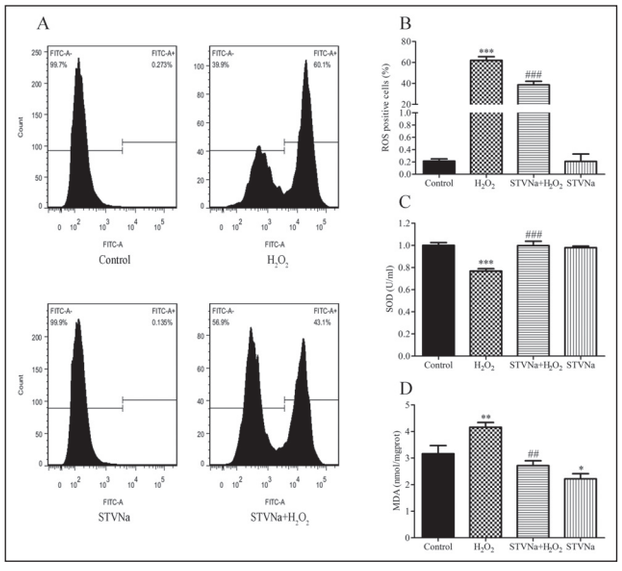

Fig. 2: Effects of STVNa on ROS, SOD activity and MDA content. (A) Original flow cytometry traces of ROS levels under control conditions and when exposed to 500 μM of H_2O_2 untreated and pretreated with STVNa (5 μM). (B-D) Graph describing ROS-positive (+FITC) cells (B), SOD activity (C) and MDA content (D) under depicted conditions. Each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the untreated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the H_2O_2 model group (n = 4).

2.3. STVNa regulates mitochondrial membrane potential and prevents apoptosis in H9c2 cells

H_2O_2 (500 μM) induced mitochondrial membrane depolarization, which was prevented by STVNa (5 μM; Figs. 3A and 3B). Additionally, we determined the levels of mitochondrial-mediated apoptosis proteins (Bcl-2 and Bax). In H_2O_2 (500 μM) group, Bcl-2 was decreased while Bax was increased and the ratio of Bcl-2/Bax was decreased (Fig. 3C). This effect of H_2O_2 was inhibited by STVNa (5 μM; Fig. 3C).

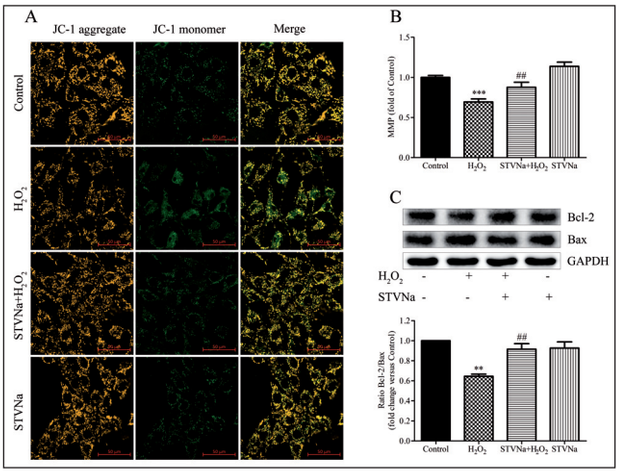

Fig. 3: Effects of STVNa on mitochondrial membranes potential (ΔΨm) and related protein levels. (A) Original images of JC-1-loaded H9c2 cells under depicted conditions and corresponding fluorescence intensity ratio graph (B). (C) Representative bands of Bcl-2, Bax and the ratio quantitative analysis results of Bcl-2/Bax. For all graphs, each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the untreated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the H_2O_2 model group (n = 3).

2.4. STVNa regulates phosphorylation of Akt and GSK-3β and promotes HK-II binding to mitochondria to protect H9c2 cells against oxidative stress

Exposure of H9c2 cells to H_2O_2 (500 μM) increased phosphorylation of Akt (ser473) while it significantly decreased phosphorylation of GSK-3β (ser9; Figs. 4A and B). Pretreatment with STVNa (5 μM) increased phosphorylation of Akt (ser473) and GSK-3β (ser9) in the presence of H_2O_2 (Figs. 4A and B). Akt further phosphorylates HK-II and facilitates its binding to mitochondria, which, in turn, promotes Bcl-2 and cardioprotection (Pastorino et al. 2005). The release of hexokinase II (HK-II) from the mitochondria initiates the opening of mitochondrial permeability transition pore (mPTP) and activation of accumulation of Bax through the active GSK-3β (Yang et al. 2017). Increased phosphorylation of GSK-3β promotes mitochondrial HK-II binding and prevents mPTP opening (Pastorino et al. 2005). We examined the levels of HK-II in the total cell and mitochondrial lysates. The level of HK-II in total cell lysates was not affected by any treatment (Fig. 4C). On the other hand, mitochondrial-bound HK-II levels were significantly reduced in H_2O_2 (500 μM) treated group (Fig. 4D) and this effect was inhibited by STVNa (5 μM; Fig. 4D). STVNa (5 μM) on its own significantly increased levels of mitochondrial-bound HK-II (Fig. 4D). LY294002 (20 μM), a PI3K inhibitor, abolished the protective effects of STVNa (5 μM) against H_2O_2 (500 μM; Fig. 4E). Immunoblotting data showed LY294002 (20 μM) pretreatment significantly inhibited STVNa-induced increase in phosphorylation of Akt (ser473; Figs. 4F and 4G) and significantly decreased phosphorylation of GSK-3β (ser9; Figs. 4F and 4H).

3. Discussion

Oxidative stress is a consequence of the imbalance between ROS production and antioxidant defence systems in the body that leads to the damage of proteins, lipids and DNA and it is important element of major myocardial pathologies including ischaemia-reperfusion, atrial fibrillation and heart failure (Betteridge 2000; Schieber et al. 2014; Zorov et al. 2014).

We have previously shown that STVNa protects the heart against ischaemia-reperfusion and improves heart function in heart failure, but this is the first report to demonstrate that STVNa protects against oxidative stress as well. In oxidative stress, increase in ROS levels leads to the damage of cellular components, decreases ATP levels and increases intracellular calcium levels. Antioxidant defence system contracting ROS is essential in restoring normal cellular functions

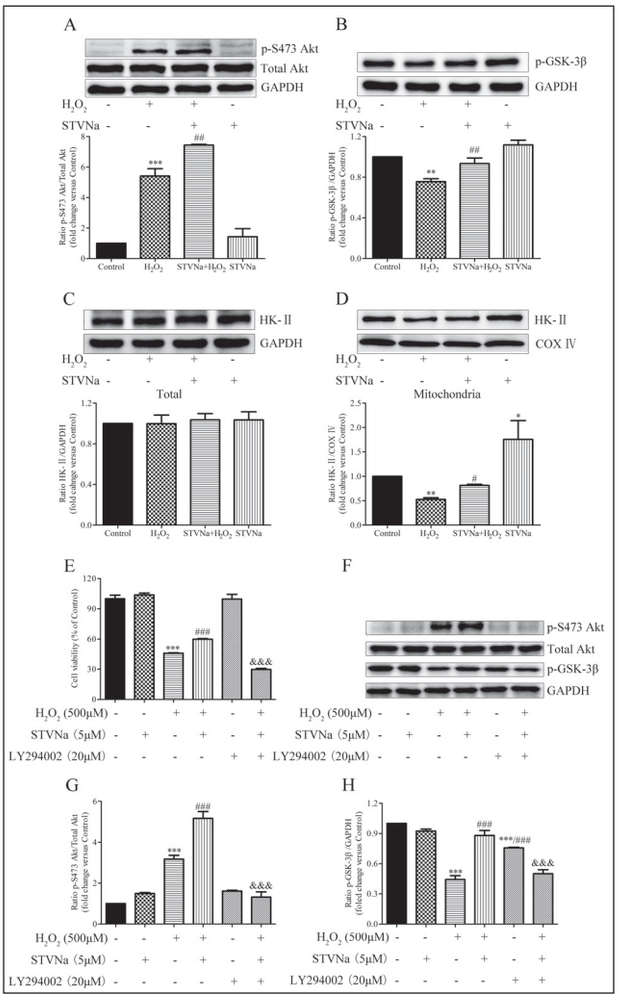

Fig. 4: Effects of STVNa on Akt/GSK-3β signalling pathway and HK-II binding to mitochondria. (A) Representative bands of p-S473 Akt, total Akt, GAPDH and the ratio of p-S473 Akt/Total Akt. (B) Representative bands of p-GSK-3β, GAPDH and the ratio of p-GSK-3β/GAPDH. (C) Representative bands of HK-II, GAPDH and the ratio of HK-II/GAPDH in the cell lysis. (D) Representative bands of HK-II, COX IV and the ratio of HK-II/ COX IV in the mitochondria. (E) Viability of H9c2 cells under depicted conditions. (F) Representative bands of p-S473 Akt, total Akt, p-GSK-3β and GAPDH. (G) Representative the ratio of p-S473 Akt/Total Akt. (H) Representative the ratio of p-GSK-3β/GAPDH. For all graphs, each bar represents mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the untreated control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the H_2O_2 model group; $^{\&\&\&}P < 0.001$, compared with the H_2O_2 model group that was pretreated with STVNa (5 μM) (n = 3).

(Poljsak et al. 2013). Here, pretreatment with STVNa reduced ROS level during oxidative stress and promoted cellular viability as determined by battery of different tests. We have also measured MDA levels as a marker of oxidative stress as this compound is obtained during the process of lipid peroxidation, as well as SOD, an antioxidant enzyme (Ayala et al. 2014). Our results showed that, STVNa pretreatment markedly decreased MDA and significantly increased SOD activity was significantly increased. Taken all together, these results suggest that STVNa protects H9c2 cells against oxidative stress, at least in part, by restoring biological antioxidant defence system in H9c2 cardiomyocytes.

When oxidative stress is induced, intracellular ROS levels increase and it is correlated with the occurrence of cells death (Schieber et al. 2014; Zhang et al. 2018) Mitochondrial ROS overload could activate both cell death pathways, i.e. necrosis and apoptosis (Kitsis et al. 2010). Necrosis is recognised as a consequence of leaking inner mitochondrial membrane, whereas apoptosis is activated due to permeabilization of the outer mitochondrial membrane and release of apoptogens (Kung et al. 2011; Sack et al. 2017). Apoptosis and necrosis affect cell survival under normal physiological conditions as well as in various diseases including myocardial infraction and reperfusion injury (Bennett 2002). Our results revealed that STVNa pretreatment significantly decreased the apoptotic and necrotic ratio. These findings further point out that STVNa protects H9c2 cardiomyocytes against oxidative stress-induced death. This conclusion is also supported by findings that STVNa improves nuclear morphology of H9c2 cells under stress conditions.

In our previous work, we reported that STVNa regulates mitochondrial fission proteins to restore mitochondrial function (Sun et al. 2018). Here, we showed that STVNa inhibits mitochondrial mebrane depolarization induced by H_2O_2 and as well as increase in Bax/Bcl-2 ratio suggesting that STVNa restores mitochondrial function to prevents from cellular apoptosis and necrosis.

It is well established that activation of Akt signaling pathway mediates cardioprotection (Miyamoto et al. 2009). Akt may prevent cell death by phosphorylating its specific targets, which alters the function and/or subcellular localizations of proteins including Bad [Bcl-2 associated agonist of cell death] and Bax (Miyamoto et al. 2009). In addition, Akt can translocate to the mitochondrial matrix and inhibit mPTP opening, thereafter inhibit cell death (Miyamoto et al. 2008). In this study, we found that STVNa activates Akt signaling pathway which was in correlation with increased levels of Bcl-2 and downregulation of pro apoptotic protein (Bax) levels, suggesting that the cytoprotective mechanism of STVNa is likely mediated through Akt signaling pathway.

It has been suggested that Glycogen synthase kinase-3β (GSK-3β) and HK-II mediate the cardioprotective effect of Akt activation (Miura et al. 2010; Roberts et al. 2013; Wu et al. 2011; Yang et al. 2017; Yang et al. 2019). Akt could directly phosphorylate GSK-3β at Ser9 to negatively regulate its kinase activity and while the active form of GSK-3β may phosphorylate VDAC1 on threonine 51 leading to reduced HK-II binding to mitochondria (Pastorino et al. 2005). Inactivation of GSK-3β by Akt thereby preserves the integrity of mitochondria (Ohori et al. 2008). In addition, Akt could also phosphorylate HK-II at Thr-473, resulting in increased accumulation of mitochondrial HK-II and protect cells against ROS induced damage (Roberts et al. 2013; Wu et al. 2011). The binding of HK-II to mitochondria inhibits mPTP opening mainly through stabilizing contact sites of outer-mitochondrial membrane (OMM) and inner-mitochondrial membrane (IMM) and interacting with OMM proteins such as Bcl-2 family members, which plays an important role in the resistance mitochondria-mediated cells death (Halestrap et al. 2015). In the present study, STVNa increased the p-GSK-3β and mitochondrial HK-II downregulated by H_2O_2. LY294002, an inhibitor of PI3K which inhibits Akt, activity, abolished protective effects of STVNa by preventing increased Akt and GSK-3β phosphorylation during H_2O_2 induced oxidative stress in H9c2 cardiomyocytes. Our results suggest that STVNa activates the Akt/GKS-3β signalling cascade to prevent of mitochondrial-mediated cell death and, consequently, confer cardioprotection against oxidative stress.

In conclusion, this is the first report to demonstrate that STVNa reduces ROS levels, preserve mitochondrial function, restore biological antioxidant defence system and prevent cell death by the activation of Akt/GSK-3β signalling and further enhancing binding of HK-II to mitochondria.

4. Experimental

4.1. Cell culture and treatment

H9c2 cardiomyocytes were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum (FBS, Gibco), penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) (Gibco, USA) under an atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were seeded on cell culture dish or well plates for 48 h before experiments to achieve 90% confluence. Then, the cells either exposed to relevant hydrogen peroxide (H_2O_2) concentrations for 2 h or pretreated with STVNa for 24 h before the H_2O_2 treatment.

4.2. Cell viability assay

Cell viability was assessed using a cell-counting kit-8 (CCK-8, Beyotime, China). Briefly, H9c2 cardiomyocytes were plated in a 96-well plate at 1×10^4 cells/well. When the cells grow to about 90% confluence, the cells were treated with H_2O_2 at

various concentrations (100, 200, 300, 400, 500 μM) for 2 h either with or without STVNa (1, 3, 5 μM) and the PI3K inhibitor LY294002 (20 μM) was used in this study. After exposed to H_2O_2, the culture medium was removed and freshly prepared 10% CCK-8 solution was added to each well and incubated for 2 h at 37 °C. Immediately after incubation, absorbance of each well was measured at 450 nm using a microplate reader (TriStar² LB 942, Berthold Technologies, Germany).

4.3. Lactate dehydrogenase (LDH) release assay

LDH release was measured using corresponding LDH cytotoxicity assay kit (Beyotime, China). The H9c2 cells were treated as described previously. After H_2O_2 treatment, 120 μL of the cell culture supernatant from each well was moved to a new plate. LDH reaction mix (60 μL) was added to each well and incubated in dark for 30 min at 25 °C. Finally, the absorbance was measured at 490 nm using a microplate reader (TriStar² LB 942, Berthold Technologies, Germany).

4.4. Detection of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined by the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) method. Briefly, the H9c2 cardiomyocytes were plated on six-well plate and pretreated with or without 5 μM STVNa for 24 h, after which they were exposed to 500 μM H_2O_2 for 2 h. The cells were rinsed twice using PBS (Gibco, USA) and incubated with 2 μM of DCFH-DA for 30 min at 37 °C. After washing the excess DCFH-DA, cells were collected with 0.25% trypsin (free from EDTA and phenol red). The cells were washed and re-suspended in 300 μL PBS and the fluorescence intensity was assessed using flow cytometer (FACSCelesta 3, BD Biosciences, USA). The data was analysed using flowjo software (version 7.6.1) and the intracellular ROS levels were expressed as percentage of FITC positive cells.

4.5. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) concentration assay

H9c2 cardiomyocytes were seeded in six-well plates at a density of 1×10^6 cell/well and treated as described previously. Then, the cells were collected, washed and centrifuged at 300×g for 10 min. The supernatant was discarded and the cells obtained through centrifugation was re-suspended in 300 μL PBS and lysed at 4 °C. SOD activity and MDA content were measured using (SOD assay kit (WST-1 method) and MDA) assay commercial kits (Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions. Protein content of the lysates were measured using bicinchoninic acid (BCA) assay (Servicebio, China).

4.6. Nuclear staining

For 4',6-diamidino-2-phenylindole (DAPI) (Servicebio, China) staining, the H9c2 cardiomyocytes were rinsed twice with ice cold PBS and then treated with cell strong fixative (Beyotime, China) for 30 min at room temperature. Then, cells were rinsed with PBS and incubated with 1 μM DAPI for 10 min at room temperature. Following incubation period, cells were rinsed and photographed using a confocal laser scanning microscopy (LSM800, Zeiss, Germany).

4.7. Cell apoptosis and necrosis assay

Annexin V/PI fluorescein staining kits (Bestbio, Shanghai, China) were used to determine apoptosis and necrosis ratio in H9c2 cardiomyocytes. Briefly, the cells were prepared as previously described. Cells were collected, rinsed and re-suspended in 400 μL of binding buffer solution and incubated with Annexin V solution for 15 min. Cells were then incubated with PI solution for 5 min in the dark and apoptosis and necrosis ratio was detected by flow cytometry (FACSCelesta 3, BD Biosciences, USA).

4.8. Mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential of H9c2 cell was measured using JC-1 dye (Jiancheng Bioengineering Institute, China). Cells were seeded into dishes and treated as previously described. The cells were then incubated in JC-1staining solution (5 mg/ml) for 20 min at 37 °C. Subsequently, the stained cells were washed twice with JC-1 staining buffer and images were taken by a confocal laser scanning microscopy (LSM800, Zeiss, Germany).

4.9. Isolation of mitochondria

Mitochondria were isolated using a mitochondrial protein extraction kit (BestBio, Shanghai, China) in accordance with the manufacturer's instructions. In brief, cells were harvested after centrifugation at 500×g for 5 min at 4 °C and then washed with ice-cold PBS. After adding solution-A from the kit, the cells were kept in the 4 °C for 5 min followed by full homogenization with an ultrasonic cell homogenizer. After centrifugation at 500×g for 5 min at 4 °C, the supernatant was collected, transferred to an ice-cold tube, and centrifuged at 1000×g for 10 min at 4 °C. Subsequently, the supernatant was transferred to newly labelled tube and centrifuged at 10,000×g for 20 min at 4 °C. The precipitate was resuspended with solution B and then centrifuged at 10,000×g for 20 min at 4 °C. Finally, the precipitate was resuspended in solution C and contained the mitochondrial proteins.

4.10. Western blot analysis

For Western blotting, H9c2 cells were harvested using RIPA lysis buffer containing protease inhibitors (Servicebio, China). The lysates were collected, vortexed and centrifuged at 4 °C for 10 min at 13000 x g to remove cell debris. The supernatant was then snap frozen and stored at -80 °C until further use. Bicinochoninic acid (BCA) assay (Servicebio, China) was used to measure the protein concentrations in the samples. For each sample (20 μg) of protein was used to perform SDS-PAGE and transferred to PVDF membrane. For all blots, overnight incubation at 4 °C was performed for primary antibodies. The antibodies includedBcl-2 (1:2000) (60178-1-lg, Proteintech, China), Bax (1:2000) (60267-1-lg, Proteintech, China), p-S473 Akt (1:1000) (BM4838, Boster Biological Technology, USA), Total-Akt (1:3000) (#4691, Cell signaling technology, USA), p-GSK-3β (1:2000) (#9323, Cell signaling technology, USA) HK-II(1:2000) (#2867, Cell signaling technology, USA) COX IV (1:2000) (#4850, Cell signaling technology, USA) and GAPDH (1:3000) (60004-1-lg, Proteintech, China). GAPDH or COX IV served as internal control. All the blots were incubated in TBST with 5% skimmed milk and either phospho or total protein determination was made using horse-radish peroxidase conjugated secondary antibodies [anti-rabbit IgG (SA00001-2, Proteintech, China) or anti-mouse IgG (SA00001-1, Proteintech, China)] and enhanced chemiluminescence reagent (Servicebio, China). The band intensities were analysed using image lab software (version 3.0) (Bio-Rad). All the blots were performed in triplicates.

4.11. Statistical analysis

Data weres analysed using a one-way analysis of variance (ANOVA) using Prism 5 (GraphPad Software Inc., San Diego, USA). All the results were expressed as means±SEM. $P < 0.05$ was considered statistically significant.

Authors' contributions: X.Z., Z.L., K.S.M.A., C.M., K.S.T. and A.J. contributed to the acquisition, analysis, interpretation of data and contributed to writing of the manuscript. W.T. initiated, designed and supervised the study, analysed the data and involved in drafting the manuscript. All authors have read and approved the final version of the paper.

Acknowledgments: This work was supported by Science and Technology innovation Project of Foshan (Grant No. 2017IT100162), Major Science and Technology Project grant of Guangdong Province (Grant No. 2014B020210001), National Natural Science Foundation of China (Grant No. 81803747) and China Postdoctoral Science Foundation (Grant No. 2018M643027). AJ was supported by the University of Nicosia Medical School. We are grateful to Key-pharma Biomedical Inc. for supplying STVNa.

Conflict of interest: The authors confirm that there is no conflict of interest.

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