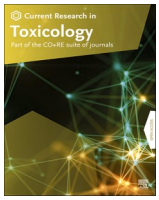
Current Research in Toxicology 5 (2023) 100136

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



Activation of PI3K/Akt mediates the protective effect of diallyl trisulfide on doxorubicin induced cardiac apoptosis

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ARTICLE INFO

Keywords:
Dially trisulfide
Doxorubicin
Cardiac apoptosis
Reactive oxygen species

ABSTRACT

Diallyl trisulfide (DATS), an organosulfide compound derived from garlic, is renowned for its potent antioxidant properties, particularly in countering the generation of reactive oxygen species (ROS). It has also gained recognition as a potential agent for preventing heart-related conditions. Doxorubicin (Dox), a commonly used chemotherapeutic drug, is known to induce severe cardiac complications by promoting ROS production. Therefore, it was imperative to investigate whether DATS possesses cardioprotective capabilities against Dox-induced cardiac apoptosis and elucidate the underlying mechanisms. In this study, we observed that the intracellular ROS levels and cardiac apoptosis were heightened in H9c2 cells exposed to Dox (1 μM). However, treatment with 10 μM DATS effectively mitigated the Dox-induced ROS generation and apoptotic signaling, concurrently activating the PI3K/Akt pathway. Notably, the anti-apoptotic effects of DATS were attenuated when PI3K siRNA and the LY294002 PI3K inhibitor were employed. Furthermore, the TUNEL assay results demonstrated a significant reduction in Dox-induced apoptosis with DATS treatment. In summary, our findings indicate that DATS can activate the PI3K/Akt pathway, reducing ROS production in cardiac cells exposed to Dox, and subsequently rescue cardiac cells from apoptosis.

Introduction

Doxorubicin (Dox) is a chemotherapeutic agent renowned for its efficacy in suppressing cancer cell proliferation. Nevertheless, its administration is often accompanied by detrimental effects on the cardiovascular system. These effects are thought to be due to the production of quinone free radicals generated during the redox cycle and the formation of reactive oxygen species (ROS) during topoisomerase II-mediated DNA damage (Zhu et al., 2016). In addition, it was demonstrated in a series of studies that intervention with ROS scavengers failed to prevent the negative implications arising from the administration of Dox (Kornfeld et al., 2015). Studies have shown that at an accumulative dose, the rate of suffering from myocarditis–pericarditis syndrome would be increased (Faridvand et al., 2020). Cardiomyopathy, followed by myocardial infarction, has been suggested as the most severe adverse effect of the long-term treatment with doxorubicin (Jenca et al., 2021). Therefore, strategies aimed at enhancing cardiac viability are essential for improving heart disorders in cancer patients undergoing treatment with Dox.

Apoptosis is a fundamental cellular process that exerts a critical role in regulating tissue homeostasis during development and maintenance. However, evidence indicates that cardiac apoptosis can initiate cascades

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https://doi.org/10.1016/j.crtox.2023.100136
Received 14 July 2023; Received in revised form 9 November 2023; Accepted 10 November 2023
Available online 11 November 2023

of events that ultimately contribute to the development of heart failure (Goldblatt et al., 2021). The release of cytochrome *c* from mitochondria, a process governed by the B-cell lymphoma 2 (Bcl-2) protein family, constitutes a critical stage in the apoptotic cascade. Phosphorylation of Bcl-2 associated agonist of cell death (BAD), a member of the Bcl-2 family that serves as a negative regulator of apoptosis, by the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, is imperative for the inhibition of apoptotic signaling (Liu et al., 2020). In its unphosphorylated state, BAD forms complexes with Bcl-2 or Bcl-XL, leading to inhibition of their anti-apoptotic function (Lu et al., 2021). Dox-induced excessive generation of ROS can lead to damage and death of cardiomyocytes. Prolonged administration of Dox can induce various factors that contribute to cardiac impairment, including cardiac hypertrophy and the development of heart failure (Rawat et al., 2021). Therefore, it is crucial to protect cardiomyocytes from oxidative stress-induced damage. Natural products that can activate the cardiac PI3K/Akt signaling pathway may serve as potential therapeutic agents against Dox-induced cardiac disorders.

Garlic (*Allium sativum*) is one of the most commonly used food additives worldwide not only because of its added flavor but also because of its antihypertension, antiatherosclerotis, anticancer, and chemoprevention properties (Hsieh et al., 2020). Diallyl trisulfide (DATS), an important oil-soluble organosulfur component and the most potent antioxidant in garlic oil, has been reported to inhibit the proliferation of various human cancer cells, including those originating from colon, lung, skin, and breast cancers (Zhang et al., 2020). Modern pharmacological studies have claimed that DATS has antiinflammation and antioxidant properties (Tsai et al., 2013; Kuo et al., 2013; Lin et al., 2021). A study of diabetic rats treated with garlic oil revealed that the activated PI3K/Akt/Nrf2-Keap1 signaling could alleviate cardiac oxidative stress (Padiya et al., 2014; Ou et al., 2010). Apart from DATS, diallyl disulfide (DADS) is also a major component in garlic oil, and has also been reported to modulate JNK and PI3K/Akt signaling in human prostate carcinoma cells (Shin et al., 2012). In this study, our objective was to elucidate the role of the PI3K/Akt signaling pathway in mediating the cardioprotective effects of DATS against Dox-induced cardiotoxicity.

Materials and methods

Cell culture and treatment

The H9c2 cells (embryonic BD1X rat cardiac myoblast) were purchased from ATCC (ATCC®CRL-1446TM). Cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10 % cosmic calf serum (Gibco), 2 g/L sodium bicarbonate, 1 % Penicillin-Streptomycin (Pen/Strep; 1U/ml; 100 μg/ml streptomycin; Corning), and incubated at 37 °C with 5 % humidified CO_2. Experiments were set up with 80 % confluent cells sub-cultured in 100-mm dishes, 60-mm dishes or 6-well plates. H9c2 cells were initially exposed to Dox for 2 h, followed by subsequent treatment with varying concentrations of DATS (1, 5, 10 μM) for a total duration of 22 h. The cells were analyzed after a total of 24 h.

Cell viability assay

The viability of H9c2 cells after treatment was assessed using the MTT assay. A total of 5x10^5 H9c2 cells were seeded into 6-well plates. After 24 h, cells were exposed to Dox for 2 h, followed by subsequent treatment with varying concentrations of DATS (1, 5, 10 μM) for a total duration of 22 h. The cells were analyzed after a total of 24 h. Subsequently, the culture medium was replaced with 1 mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/mL; prepared by diluting a 5 mg/mL stock solution in PBS with culture medium to a final concentration of 0.5 mg/mL) and incubated for 2 h at 37 °C. The purple-colored formazan product was then solubilized in 100 μL of dimethyl sulfoxide (DMSO), and the optical density at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).“

Antibodies and reagents

The following antibodies were used in this study: anti-Akt (sc-5298, Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-Akt (Ser473, #4060T, Cell Signaling), anti-Bax (sc-7480, Santa Cruz Biotechnology), anti-Bcl-2 (610539, BD), anti-Bcl-xL (sc-8392, Santa Cruz Biotechnology), anti-β-actin (sc-47778, Santa Cruz Biotechnology), cleaved caspase-3 (#9664, Cell Signaling), anti-PI3K (sc-365404, Santa Cruz Biotechnology), anti-phospho-PI3K (AF3242, Affinity Biosciences), and anti-cytochrome *c* (sc-13560, Santa Cruz Biotechnology). All secondary antibodies (anti-rabbit, anti-mouse, and anti-goat, HRP-conjugated) were purchased from Santa Cruz Biotechnology. H_2O_2, LY294002, and si-RNA-PI3K were obtained from Sigma (MO, USA).

Western blot analysis

Western blot analysis was conducted according to previously described methods (Chang et al., 2021; Shojaei Shad and Haghighi, 2018). The cultured cells were harvested and washed with PBS to remove the medium. Subsequently, the cells were lysed in RIPA buffer and kept on ice for 30 min. The lysates were then collected by centrifugation at 12,000g for 15 min at 4 °C. Protein quantification was performed, and equal amounts of 40 μg protein were loaded onto SDS-PAGE gels. The proteins were separated using 8–12 % SDS-PAGE and transferred onto PVDF membranes (Millipore, Belford, Massachusetts, USA). The membranes were blocked with 5 % non-fat dry milk in TBST for at least 1 h at room temperature. Following the blocking step, the membranes were incubated overnight at 4 °C with the appropriate primary antibodies diluted in TBST. After the primary antibody incubation, the membranes were washed to remove unbound antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, or anti-goat) diluted in TBST.

DAPI staining and TUNEL assay

DAPI staining and TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labeling) assay were performed following previously described methods, utilizing an In Situ Cell Death Detection Kit (Roche) (Lin et al., 2021). Cultured cells were fixed with a 4 % paraformaldehyde solution for 30 min at room temperature, followed by PBS washing and permeabilization with a solution containing 0.1 % Triton X-100 in 0.1 % sodium citrate for 2 min. Subsequently, the cells were incubated with a TUNEL reagent to detect DNA fragmentation indicative of apoptotic cells. For DAPI staining, the cells were treated with 1 μg/ml DAPI reagent for 3 min to visualize the cell nucleus. Images were captured using a Leica SP2 confocal spectral microscope.

Reactive oxygen species production

Intracellular generation of ROS was assessed using flow cytometry with the peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). The experimental protocol entailed incubating cells with 5 μM DCFH-DA, which was prepared by diluting 5 mM DCFH-DA stock solution in DMSO. This incubation was carried out at 37 °C for a duration of 30 min, and as a positive control, 200 μM H_2O_2 was added. DCFH-DA was enzymatically converted to DCFH intracellularly, and upon oxidation by ROS, it formed the fluorescent product dichlorofluorescein (DCF). Flow cytometric analysis was conducted employing a BD FACS Canto flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) to quantify intracellular ROS production. The selection of viable cells was accomplished by applying a gating strategy through FCM analysis, which involved two steps: initially, utilizing the Forward Scattered Light (FSC) versus Side Scattered Light (SSC)

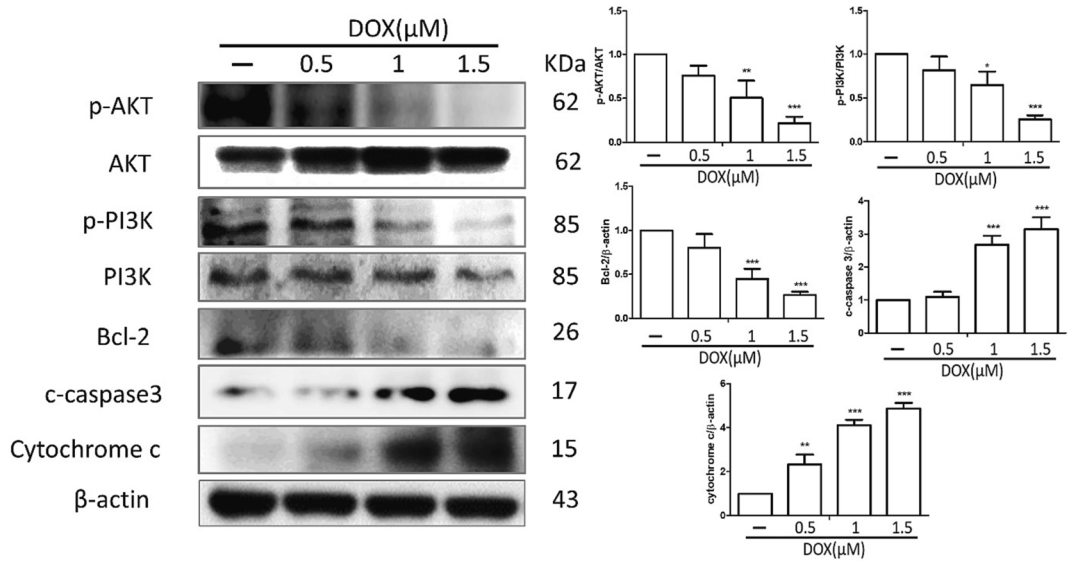

Fig. 1. Dox induces the apoptosis of cardiac cells. Cells were treated with Dox at concentrations of 0.5, 1, and 1.5 μM for 24 h. The expression of PI3K/Akt, Bcl-2, and caspase-3 were evaluated by western blot analysis. β-actin was used as a loading control. Values shown are means ± SD. Quantification of the results is shown (n = 3); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control cells.

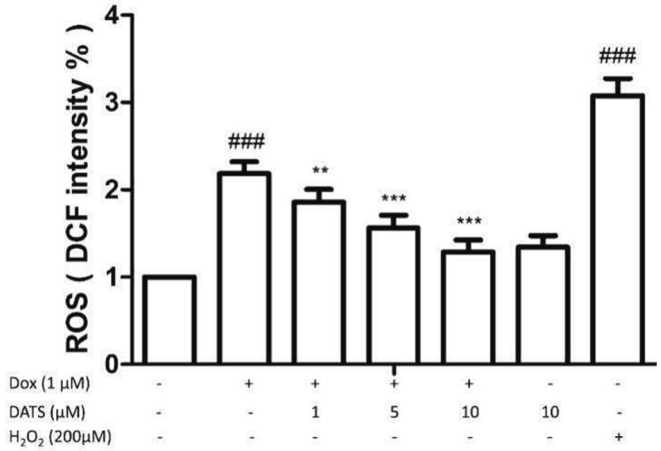

Fig. 2. DATS exhibited a dose-dependent reduction in the doxorubicin-induced generation of ROS. H9c2 cells were initially exposed to 1 μM Dox for a duration of 2 h, followed by treatment with DATS at different concentrations (1, 5, and 10 μM) for an additional 22 h. Cellular ROS was detected using the dichlorofluorescein (DCF) assay for 15 min and examined by flow cytometry. Values shown are means ± SD. Quantification of the results is shown (n = 3); $^{**}p < 0.01$, $^{***}p < 0.001$ versus Dox-treated cells; $^{###}p < 0.001$ versus control.

parameters, and subsequently, employing DCFH-DA staining (FITC). This conventional gating approach relies on the optical signals (FSC/SSC) acquired by the cytometer. Each treatment was executed in triplicate to ensure the reproducibility of results.

Statistical analysis

The experiments were performed in triplicate, and the data were analyzed using the statistical software GraphPad Prism5 (San Diego, CA, USA). Statistical analysis was conducted using appropriate tests such as one-way ANOVA followed by Tukey's or Dunnett's post hoc tests to compare the means of multiple groups. The quantitative data are presented as the mean ± standard deviation (SD) based on three or more replicates. A p-value of less than 0.05 was considered statistically significant. The results from the Western blot analysis were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Results

Doxorubicin-induced cardiac apoptosis

The results obtained from western blot analysis demonstrated that Dox treatment resulted in an upregulation of apoptosis-related proteins and a downregulation of p-PI3K/Akt proteins in cardiac cells. Following a 24-hour exposure to Dox at concentrations of 0.5, 1, and 1.5 μM, there was a dose-dependent increase in the expression of apoptosis-related markers, including c-caspase 3 and cytochrome *c* proteins. Concurrently, the activation of AKT/PI3K signaling showed a dose-dependent reduction (Fig. 1). These findings provide evidence that treatment with Dox induces apoptosis in cardiac cells.

Diallyl trisulfide exhibited a dose-dependent reduction in the doxorubicin-induced generation of ROS

H9c2 cells were initially exposed to 1 μM Dox for a duration of 2 h, followed by treatment with DATS at different concentrations (1, 5, and 10 μM) for an additional 22 h. Our results demonstrated that DATS dose-dependently reduced Dox-induced ROS generation. This suggests that DATS may protect against Dox-induced oxidative stress injury by mitigating ROS production in cardiac cells (Fig. 2).

Diallyl trisulfide increased the survival of cardiac cells via the PI3K/Akt pathway

To examine the potential regulatory effect of DATS on the PI3K/Akt pathway, we subjected cardiac cells to various concentrations of DATS (1, 5, and 10 μM) for 24 h. Subsequently, we performed western blot analysis to evaluate the protein levels associated with the PI3K/Akt pathway. Our results demonstrated that DATS treatment led to the upregulation of PI3K/Akt pathway-related proteins in cardiac cells (Fig. 3A).

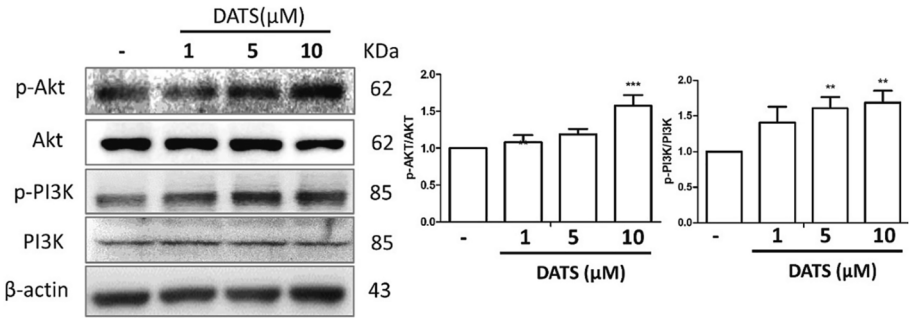

Fig. 3. DATS increases the survival of cardiac cells. Cells were treated with DATS at the indicated concentrations (1, 5, and 10 μM) for 24 h. The expression of apoptotic and survival proteins was examined by western blot analysis. β-actin was used as a loading control. Values shown are means ± SD. Quantification of the results is shown (n = 3); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control cells.

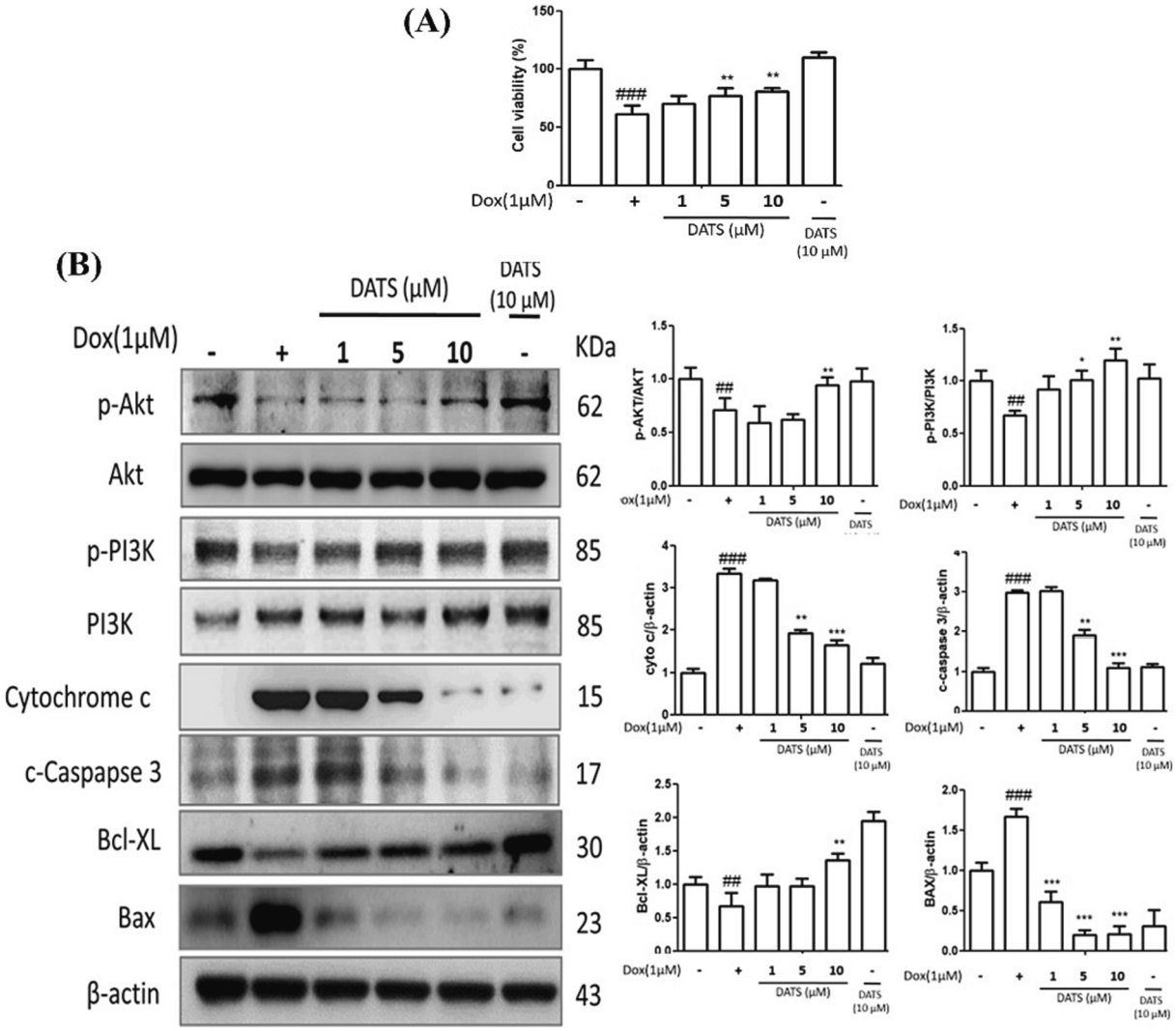

Fig. 4. DATS inhibits apoptosis by decreasing the mitochondrial pathway in cardiac cells following exposure to Dox. H9c2 cells were initially exposed to 1 μM Dox for a duration of 2 h, followed by treatment with DATS at different concentrations (1, 5, and 10 μM) for an additional 22 h. Cells only treated with 10 μM of DATS for 24 h were used as a positive control. (A) Cell viability was examined using an MTT assay. (B) Protein expression was evaluated by western blot analysis. β-actin was used as a loading control. Values shown are means ± SD. Quantification of the results is shown (n = 3); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Dox-treated cells; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus control.

Diallyl trisulfide inhibited doxorubicin-induced cardiac apoptosis through the PI3K/Akt pathway

Cell viability was assessed using the MTT assay. The data suggest that DATS treatment significantly attenuated the reduction in cardiac cell viability induced by Doxorubicin (Dox). Our findings provide evidence that treatment of H9c2 cells with DATS led to the activation of PI3K/Akt signaling pathway, which is associated with cell survival. Moreover,

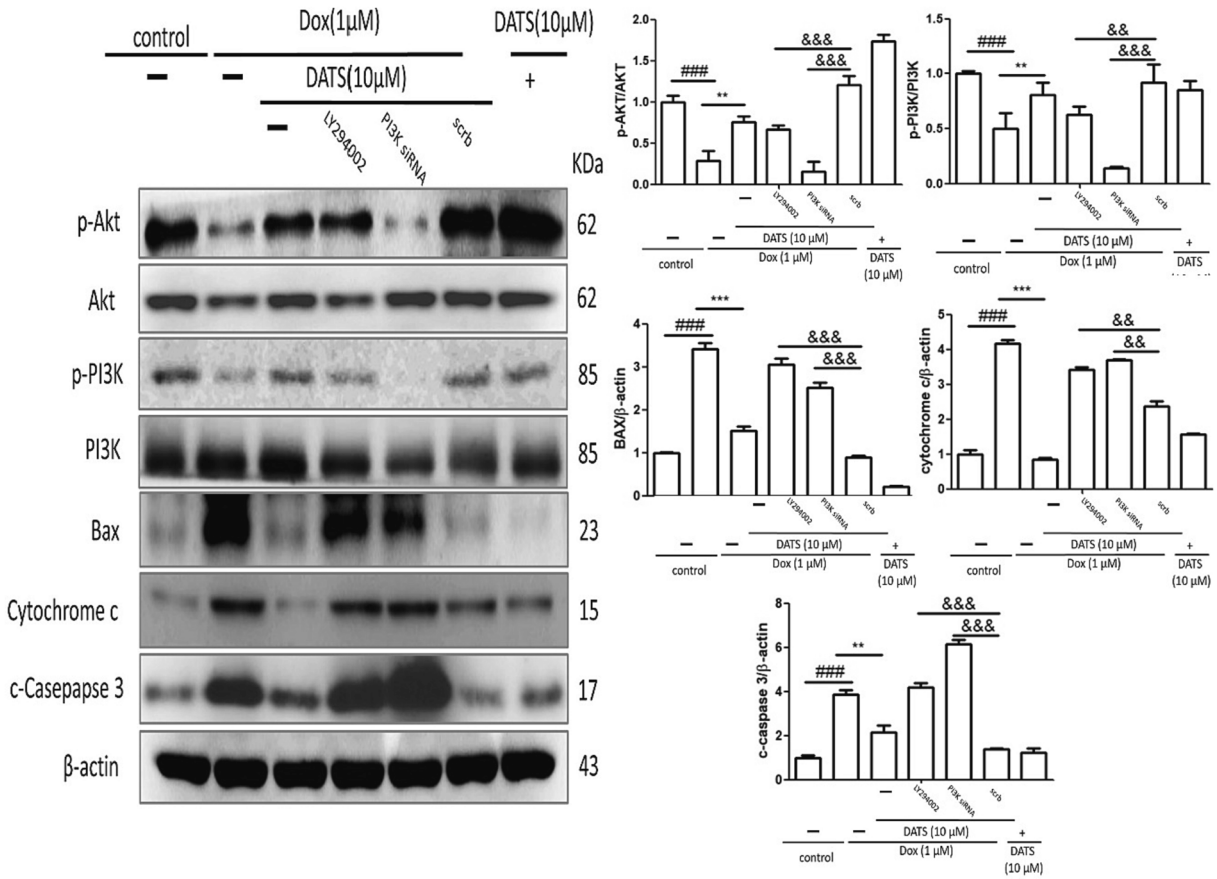

Fig. 5. DATS suppresses Dox-induced apoptosis through the activation of the PI3K/Akt signaling pathway. H9c2 cells were initially exposed to 1 μM Dox for a duration of 2 h, followed by treatment with 10 μM DATS and combined with LY294002 (a PI3K/Akt inhibitor) or PI3K siRNA for an additional 22 h. Protein expression was evaluated by western blot analysis. β-actin was used as a loading control. Values shown are means ± SD. Quantification of the results is shown (n = 3); $^{**}p < 0.01$, $^{***}p < 0.001$ versus Dox-treated cells; $^{\#\#\#}p < 0.001$ versus control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ versus Dox + DATS-treated cells.

DATS treatment resulted in a decrease in apoptotic-related signaling, indicating its potential role in promoting cell survival (Fig. 4). To further investigate the involvement of the PI3K/Akt pathway in DATS-mediated cell survival, we employed PI3K inhibitors, LY294002, and PI3K siRNA. Our results demonstrated that inhibition of PI3K activity by LY294002 and siRNA-mediated knockdown of PI3K resulted in an upregulation of apoptotic-related signaling and suppression of PI3K/Akt pathway activation (Fig. 5).

Diallyl trisulfide rescued cardiac cells from apoptosis

A TUNEL assay was conducted to evaluate apoptosis in cardiac cells. Apoptotic cells were visualized by green fluorescence, while cell nuclei were stained with blue fluorescence. Our findings demonstrated an elevation in the number of apoptotic nuclei after exposure to Dox, which was mitigated upon treatment with DATS (Fig. 6A). Additionally, the anti-apoptotic effects of DATS were counteracted when PI3K activity was inhibited using siRNA or LY294002, resulting in cardiac cell death (Fig. 6B).

Discussion

Oxidative stress is known to be considerably engaged in the apoptosis of cardiomyocytes. In our study, we observed that treatment with Dox resulted in the generation of cellular ROS, which has been implicated in causing severe heart failure. Several studies have indicated that Dox can exert cardiotoxicity (Mitry and Edwards, 2016; Pei et al., 2016). Cardiomyocytes, which are rich in mitochondria, are known to be prone to oxidative damage (Gao et al., 2008). Upon encountering cellular damage, the mitochondrial pathway is activated through the release of cytochrome *c* from the mitochondria, subsequently initiating the process of apoptosis (Zimmermann and Green, 2001). In our study, we found that the Dox-induced cardiac production of ROS cause cardiac apoptosis, which was inhibited by DATS, a powerful antioxidant presence in garlic oil. Furthermore, our results of DATS reducing the apoptosis of Dox-exposed cardiac cells were reversed by treatment with PI3K siRNA, indicating that the protection provided by DATS to cardiac cells against Dox-induced apoptosis was mediated by the activation of the PI3K/Akt signaling.

Doxorubicin is an effective anticancer drug, but has been reported to lead to the generation of ROS and inhibition of topoisomerase II, causing severe side effects, such as cardiac damage. Therefore, identifying a natural compound that could be used for the treatment of the Dox-induced side effects was deemed necessary. Liu et al. demonstrated that the antioxidant effect was considered to be the most important (Liu et al., 2006). As reported in our study, DATS was shown to serve as a powerful antioxidant against the Dox-induced cardiotoxicity. These findings are consistent with our previous study, in which we demonstrated that DATS effectively mitigates the detrimental effects of high glucose-induced oxidative damage on cardiomyocytes. We observed that DATS treatment activates the PI3K/Akt pathway, thereby conferring protection against oxidative stress. Furthermore, our previous *in vivo* study indicated that oral administration of 40 mg/kg of DATS enhanced Nrf2-mediated antioxidant signaling and improved cardiac apoptosis in diabetic rats. (Tsai et al., 2013). Nonetheless, another study reported that the administration of a high dose of DADS (80 mg/kg BW)

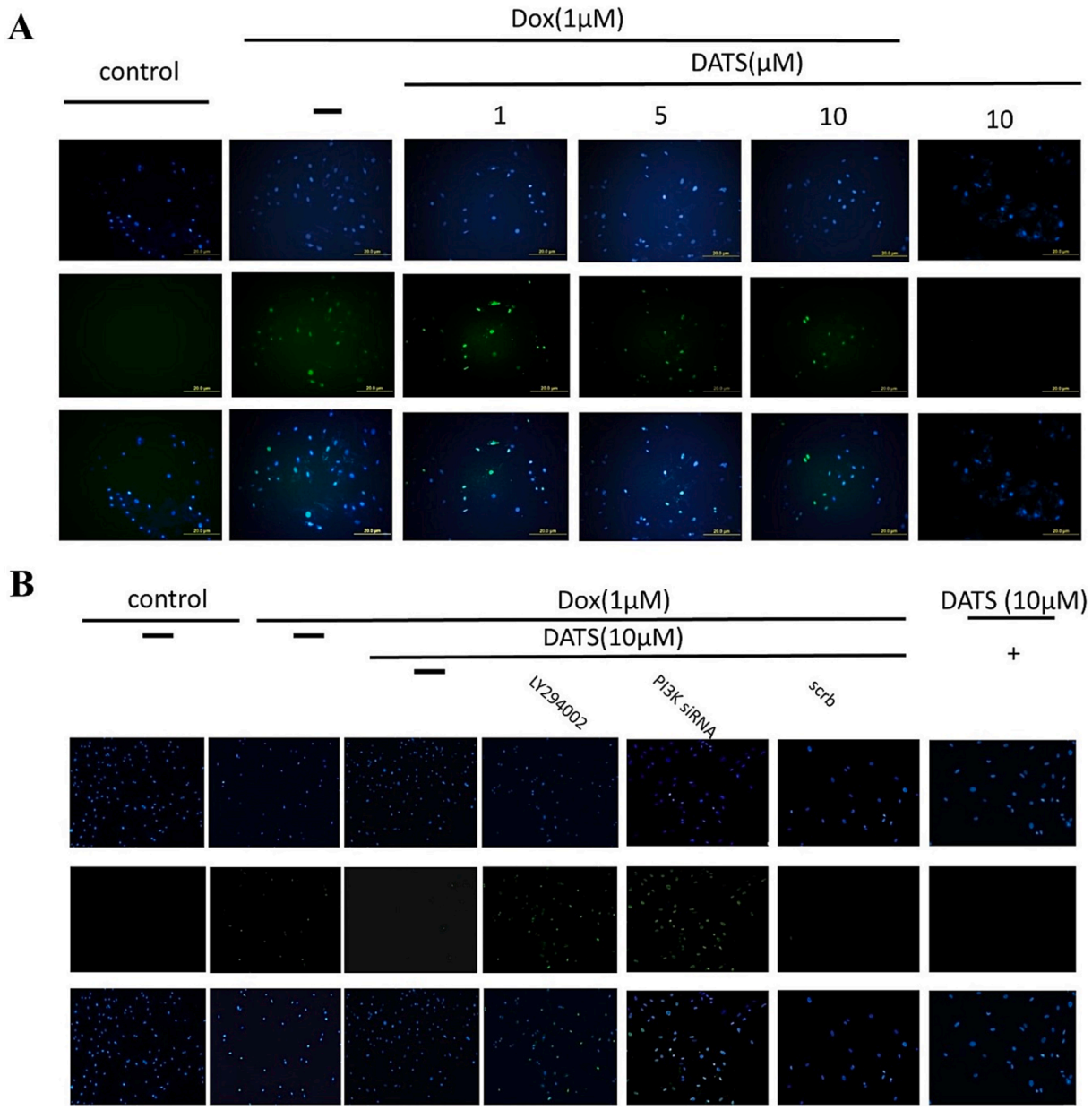

Fig. 6. Mediation of PI3K/Akt signaling in the antiapoptotic effect of DATS on the Dox-induced apoptosis of cardiac cells. Cells were treated as described in Fig. 5. Apoptosis of cells was examined by TUNEL assay; green representing apoptosis and blue (DAPI) representing cell nuclei.

resulted in a decrease in the animals' weight gain and the ratio of muscle weight to body weight compared with diabetic rats (Liu et al., 2006). This suggests that excessive DATS dosages may have adverse effects in diabetic patients; however, lower doses appear to exhibit beneficial effects.

Diallyl trisulfide, a critical component of garlic, has been long identified as a healthy food constituent, and thus was considered as a good candidate to test the possible efficacy in cardiac cells treated with Dox. Additionally, the application of natural antioxidants instead of synthesized drugs for the inhibition of oxidative stress and alleviation of Dox-induced apoptosis is a beneficial approach.

Many investigations have reported that the antioxidant effect of DATS was identified to be involved in various cell signaling pathways, such as PKC, PI3K/Akt, and MAPK related to the Nrf2 pathway. For instance, Xian et al. demonstrated the beneficial effect of DATS on scavenging free radicals; particularly, the intervention of B35 neuroblastoma cells with DATS was shown to attenuate PI3K/Akt in modulating the Nrf2/HO-1 signaling pathway against oxygen glucose deprivation (OGD)-induced apoptosis. As OGD has been reported to cause hypoxic neural injury, it has been widely used to mimic the events following an ischemic insult, and also as an in vitro model for stroke (Xu et al., 2015). In addition, many other studies have shown that DATS could activate the PI3K/Akt pathway. Using an *in vivo* model, Yi-Chang et al. found significantly increased levels of p-Akt in the group cotreated with cholesterol and garlic oil, indicating that the combination of cholesterol and garlic oil activated the PI3K-Akt/PKB pathway, thereby inhibiting the apoptosis of cardiomyocytes (Padiya et al., 2014). In our previous study, we showed that DATS increased the circulatory levels of H_2S and upregulated the levels of cystathionine gamma-lyase and p-Akt (Cheng et al., 2013; Tsai et al., 2015). Here, it was revealed that DATS downregulated the Dox-induced mitochondrial apoptotic pathway, whereas it enhanced the PI3K/Akt pathway. Based on the observation that the PI3K siRNA reversed the antiapoptotic effect of DATS following Dox-exposure, we assumed that DATS protected cardiomyocytes from apoptosis via the activation of the PI3K/Akt signaling pathway.

Conclusion

In brief, our study demonstrated that DATS protected cardiac cells from Dox-induced oxidative stress by upregulating the PI3K/Akt signaling, and concomitantly suppressing the expression of apoptotic proteins. Particular emphasis should be given to the fact that DATS could be used as a potential supportive therapy for patients undergoing treatment with Dox.

Funding

This study was supported by China Medical University (grant no. CMU109 MF-13 10951DD) and Ministry of Science and Technology-MOST (109-2320-B-039-038-MY3).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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