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Research Article

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Rhein attenuates doxorubicin-induced cardiotoxicity by regulating Drp1-mediated mitochondrial fission via the PI3K/Akt pathway

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Abstract: Mitochondrial dynamics, including mitochondrial fusion and fission, have been identified as a critical regulator of heart function. Doxorubicin (Dox) is a highly effective chemotherapeutic agent that has demonstrated broad-spectrum activity against a variety of tumor types, whereas its application is limited due to cardiotoxic effects. Rhein, a medicinal active ingredient found in rhubarb, possesses a wide range of pharmacological activities. However, whether it exerts a cardioprotective role in Dox-induced cardiotoxicity by regulating mitochondrial dynamics remains to be investigated. In this study, we found that Dox treatment promoted mitochondrial fission and elevated the expression of dynamin-related protein 1 (Drp1) in cardiomyocytes. In addition, Drp1 deletion reduced mitochondrial fission and mitochondrial ROS, and attenuated Dox-induced cardiotoxicity both in vitro and in vivo. Furthermore, rhein treatment rescued Dox-induced cardiac damage in vivo. Mechanistically, rhein attenuated Dox-induced cardiotoxicity by regulating Drp1-mediated mitochondrial fission and mitochondrial ROS in cardiomyocytes. Moreover, it alleviated the Dox-induced upregulation of Drp1 by activating phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. In conclusion, our findings demonstrate that rhein attenuates Dox-induced cardiotoxicity by regulating Drp1-mediated mitochondrial fission and mitochondrial ROS via the PI3K/Akt pathway.

Key words: Rhein; Cardiotoxicity; Mitochondrial fission; Drp1; Akt

Abbreviations

α-SMA, alpha smooth muscle actin; BAX, Bcl-2-associated x; COL1A1, collagen type I alpha 1 chain; COL3A1, collagen type III alpha 1 chain; C-CASP3, cleaved-caspase 3; Dox, doxorubicin; Dex, dexrazoxane; Drp1, dynamin-related protein 1; EdU, 5-Ethynyl-2'-deoxyuridine; FS, left ventricular fractional shortening; FOXA1, forkhead box a1; Fis1, fission protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3, histone H3; HDAC, histone deacetylase; HE, hematoxylin and eosin; LVEF, left ventricular ejection fraction; LV-Drp1, lentivirus encoding Drp1; Mfn1/2, mitochondrial fusion proteins 1/2; MMP, mitochondrial membrane potential; NR3C1, nuclear receptor subfamily 3, group c, member 1; p38 MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; P-PI3K, phosphorylated phosphoinositide 3-kinase; P-Akt, phosphorylated Akt; RXRα, retinoid x receptor alpha; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; siRNA, small interfering RNA; VDR, vitamin d receptor.

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1 Introduction

Doxorubicin (Dox), a member of the anthracycline antibiotic family, is a highly effective chemotherapeutic agent widely used in the treatment of various solid tumors and hematologic malignancies (Sturgeon et al., 2019; Wu et al., 2023). However, as many as one in four patients may develop Dox-induced cardiotoxicity, limiting its clinical application (Wu, et al., 2023). Dox-induced cardiotoxicity is characterized by cardiac enlargement with chamber dilation, a progressive decline in left ventricular ejection fraction (LVEF), and ultimately the development of heart failure (Swain et al., 2003; Quagliariello et al., 2021; Wu, et al., 2023). Despite its clinical significance, the mechanisms underlying Dox-induced cardiotoxicity remain poorly understood, and strategies for preventing such adverse reactions are limited (Wang et al., 2024). Currently, dexrazoxane (Dex) is the only FDA-approved cardioprotective agent used to prevent Dox-induced cardiotoxicity, although a recent study has suggested that Dex might increase the risk of bone marrow toxicity (Chen et al., 2024). Therefore, it is crucial to study the potential mechanisms and signaling pathways participating in Dox-induced cardiotoxicity.

Mitochondrial dynamics, namely mitochondrial fusion and fission, regulate the morphology, quantity and position of mitochondria in cells. Mitochondrial dynamics are crucial for proper cell functions, including energy production, cell movement, cell cycle, and apoptosis (Chen et al., 2023). The dysregulation of mitochondrial dynamics, characterized by dysfunctional mitochondria, is a key pathogenic mechanism of multiple conditions such as cardiovascular diseases (Chatterjee et al., 2010; Vejpongsa and Yeh, 2014; Zhang et al., 2021; Chen, et al., 2023). Excessive mitochondrial fission is commonly observed in the heart under stressful conditions, which can result in mitochondrial dysfunction and impaired cardiac performance. Mitochondrial fission is regulated by a distinct set of proteins, including dynamin-related protein 1 (Drp1) and fission protein 1 (Fis1). There have been reports indicating that ischemia causes excessive mitochondrial fission and fragmentation, resulting in cardiomyocyte death (Vejpongsa and Yeh, 2014; Pan et al., 2021; Zhang, et al., 2021; Chen, et al., 2023). Therefore, the inhibition of excessive mitochondrial fission has demonstrated therapeutic benefits across various cardiac conditions, such as diabetic cardiomyopathy and atherosclerosis (Ding et al., 2018; Jin et al., 2021). In addition to mitochondrial fission, mitochondrial fusion is essential for maintaining mitochondrial homeostasis and preserving normal cardiac function. Enhancing mitochondrial fusion through the upregulation of fusion-associated proteins has been shown to alleviate myocardial damage caused by ischemia or diabetes (Zhang et al., 2019; Liu et al., 2021).

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a bioactive compound predominantly present in rhubarb (Rheum palmatum L.), which has been extensively utilized in traditional Chinese medicine (Leask, 2015; Barbosa et al., 2020). It exhibits a wide range of pharmacological activities, including antitumor, antibacterial, anti-inflammatory, anti-Alzheimer's disease, and anti-osteoporotic properties (Gharanei et al., 2013; Zhuang et al., 2021; Maneechote et al., 2022; Sygitowicz and Sitkiewicz, 2022; Chen, et al., 2024). Recent studies have demonstrated that rhein exerts protective effects against cardiovascular diseases (Barbosa, et al., 2020; Li et al., 2022). For instance, it acted as a novel histone deacetylase (HDAC) inhibitor and demonstrated antifibrotic potential in human myocardial fibrosis (Barbosa, et al., 2020). It has been reported that rhein ameliorates cardiac hypertrophy induced by transverse aortic constriction through the regulation of the signal transducer and activator of transcription 3 (STAT3) and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways (Li, et al., 2022). A recent study has shown that rhein effectively attenuates angiotensin II-induced cardiac remodeling via the modulation of the AMPK-FGF23 signaling pathway (Lu et al., 2022). These findings underscore that rhein may play a protective role in mitigating cardiac remodeling and fibrosis. However, it remains to be elucidated whether rhein exerts cardioprotective effects against Dox-induced cardiotoxicity through the regulation of mitochondrial dynamics. In this study, we provide the first evidence that rhein

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attenuates Dox-induced cardiotoxicity by activating the Phosphoinositide 3-kinase (PI3K) /Akt signaling pathway, which in turn inhibits the nuclear translocation of nuclear receptor subfamily 3, group c, member 1 (NR3C1) and subsequently modulates Drp1-mediated mitochondrial fission.

2 Results

2.1 Dox-mediated Drp1 upregulation and mitochondrial fission in cardiomyocytes

We first established the cardiotoxicity model by treating primary cardiomyocytes with Dox, and found that the expression levels of the apoptotic proteins Bcl-2-associated x (BAX) and cleaved-caspase 3 (C-CASP3) were significantly upregulated (Fig. 1a). Besides, Dox treatment promoted the proliferation of cardiac fibroblasts (Fig. 1b). The levels of protein and mRNA expression of fibrosis markers collagen type I alpha 1 chain (COL1A1), collagen type III alpha 1 chain (COL3A1) and alpha smooth muscle actin (α-SMA) in cardiac fibroblasts were increased dramatically (Figs. 1c and 1d). These results demonstrate that the cardiotoxicity cell model was successfully constructed in vitro. Furthermore, Dox treatment led to a decrease in mitochondrial membrane potential (MMP) in cardiomyocytes and an elevation in mitochondrial ROS level (Figs. 1e and 1f). Thus, we further studied the mitochondrial dynamics in cardiomyocytes upon Dox treatment. Primary cardiomyocytes predominantly displayed elongated and interconnected mitochondrial networks in the control group, whereas the mitochondria of cardiomyocytes were smaller than 1 μm upon Dox exposure, evidenced by an increase in the number of mitochondria per cell and a decrease in mitochondrial size (Fig. 1g).

We further analyzed the relative gene expression of RNA-seq data (GSE224157) for hearts in mice injected with Dox, and found that the mitochondrial fission protein Drp1 was upregulated upon Dox treatment (Fig. 1h). To validate this result, we constructed an in vivo cardiotoxicity model by injecting mice with Dox. We observed that among the mitochondrial fission proteins, including Drp1 and Fis1, only the expression of Drp1 was significantly upregulated in hearts treated with Dox (Figs. 1i and 1j). Consistently, the expression of Drp1 was dramatically elevated in primary cardiomyocytes treated with Dox (Fig. S1a). These data indicate that Drp1-mediated mitochondrial fission was enhanced following Dox treatment in both cardiomyocytes and murine hearts.

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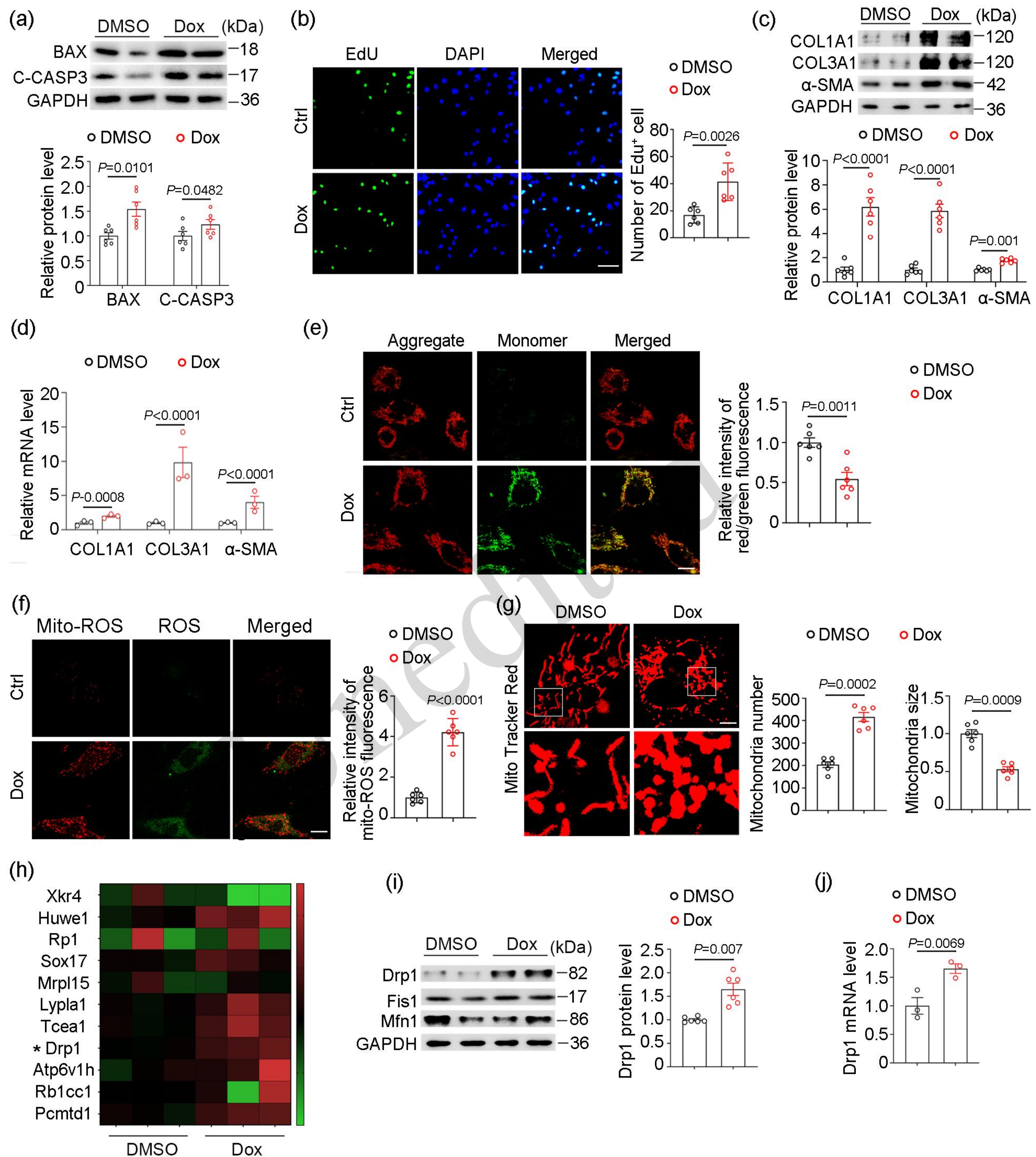

Fig. 1. Doxorubicin(Dox)-mediated Dynamin-related protein 1(Drp1) upregulation and mitochondrial fission in cardiomyocytes. (a) Primary cardiomyocytes were treated with 3 μM Dox for 24 h, and the protein levels of Bcl-2-associated X (BAX) and cleaved-caspase 3 (C-CASP3) were examined by immunoblotting (n = 6). (b) Cardiac fibroblasts were treated with 3 μM Dox for 24 h, and then fixed and stained with 5-Ethynyl-2'-deoxyuridine (EdU)-488 and DAPI to visualize the proliferating cells. Scale bar, 50 μm (n = 6). (c and d) Cardiac fibroblasts were treated with Dox, and the protein and mRNA levels of fibrosis markers were examined by immunoblotting (c) (n = 6) and RT-qPCR (d) (n = 3). (e) Cardiomyocytes were treated with Dox, and the mitochondrial membrane potential was examined by staining with JC-1. The relative intensity of

red/green was measured. Scale bar, 20 μm (n = 6). (f) Representative images of MitoSOX-stained mitochondrial ROS (red fluorescence) and DCFH-DA-stained cellular ROS production (green fluorescence). The relative mitochondrial ROS fluorescence density was measured as a fold change compared with the control group. Scale bar, 20 μm (n = 6). (g) Mitochondrial morphology was demonstrated by staining with MitoTracker Red, and the number and size of mitochondria were analyzed. Scale bar, 20 μm (n = 6). (h) Comparative gene expression profiling analysis of RNA-seq data (GSE224157) for hearts of mice treated with DMSO or Dox. (i-j) Dox (5 mg/kg) was administered to the mice by tail vein injection once a week for three weeks consecutively. The protein levels of mitochondrial dynamic molecules were examined by immunoblotting (i) (n = 6), and the mRNA level of Drp1 was detected by RT-qPCR (j) (n = 3) in mice hearts. Two-tailed unpaired Student's t test was used to compare the two groups (Figs. 1a-1g, 1i and 1j).

2.2 Drp1 inhibition-mediated alleviation of Dox-induced cardiotoxicity and mitochondrial dysfunction

To further elucidate the role of Drp1 in Dox-induced cardiotoxicity, we transfected the primary cardiomyocytes with Drp1 small interfering RNA (siRNA). Drp1 knockdown significantly increased mitochondrial size and decreased the number of mitochondria per cell in the presence of Dox, suggesting that Drp1 depletion attenuated mitochondrial fission in the Dox-treated cardiomyocytes (Fig. 2a). Besides, Drp1 depletion elevated MMP and reduced mitochondrial ROS level upon Dox treatment (Figs. 2b and 2c). Furthermore, Drp1 knockdown improved cell viability, as indicated by the decreased levels of BAX and C-CASP3 proteins (Fig. 2d). Drp1 depletion alleviated the Dox-induced toxicity of cardiac fibroblasts, evidenced by the decreased levels of fibrosis markers (Figs. 2e and 2f). These data indicate that the inhibition of Drp1-mediated mitochondrial fission mitigated Dox-induced cardiotoxicity.

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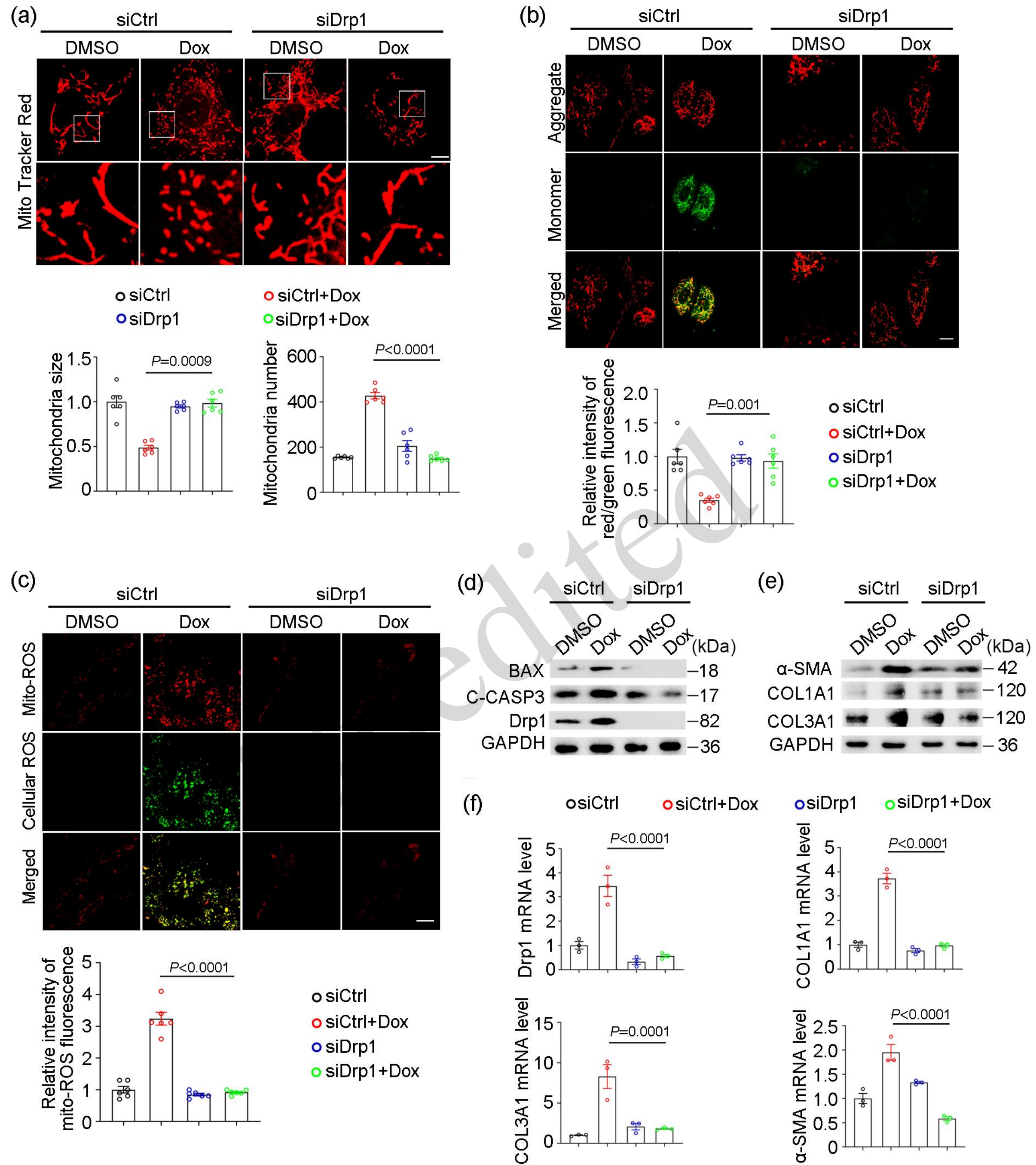

Fig. 2. Drp1 inhibition-mediated alleviation of Dox-induced cardiotoxicity and mitochondrial dysfunction. (a-d) Primary cardiomyocytes were transfected with control or Drp1 small interfering RNA (siRNA), and then treated with Dox for 24 h. (a) Mitochondrial morphology was demonstrated by staining with MitoTracker Red, and the number and size of mitochondria were analyzed. Scale bar, 20 μm ($n = 6$). (b) The mitochondrial membrane potential was examined by staining with JC-1, and the relative intensity of red/green was measured. Scale bar, 20 μm ($n = 6$). (c) The mitochondrial reactive oxygen species (ROS) and cellular ROS were stained by MitoSOX and DCFH-DA, respectively. Scale bar, 20 μm ($n = 6$). (d) Cells were collected and subjected to immunoblotting ($n = 6$). (e and f) Cardiac fibroblasts were transfected with control or Drp1 siRNA,

and then treated with Dox for 24 h. Cells were collected and subjected to immunoblotting or RT-qPCR analysis to examine the protein (e) and mRNA (f) level of fibrosis molecules (n = 3). One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. a-c and f).

2.3 Drp1 overexpression-mediated mitochondrial dysfunction and apoptosis in Dox-treated cardiomyocytes

To investigate the role of Drp1-mediated mitochondrial fission in the development of cardiotoxicity, we transfected primary cardiomyocytes with lentivirus encoding Drp1 (LV-Drp1) or lentivirus empty vector (LV-EV). The expression level of Drp1 was significantly upregulated after LV-Drp1 transfection (Figs. 3a and 3b). Drp1 overexpression inhibited cell viability, as indicated by the increased levels of apoptotic proteins BAX and C-CASP3 (Fig. 3b). Furthermore, Drp1 overexpression aggravated the Dox-induced toxicity of cardiac fibroblasts, evidenced by the increased levels of fibrosis markers (Figs. 3c and 3d). Compared with the control group, LV-Drp1 strikingly reduced mitochondrial size and increased mitochondrial number per cell, as well as reduced MMP (Figs. 3e and 3f). These results demonstrate that Drp1 overexpression enhanced mitochondrial fission and contributed to apoptosis in cardiomyocytes following Dox treatment.

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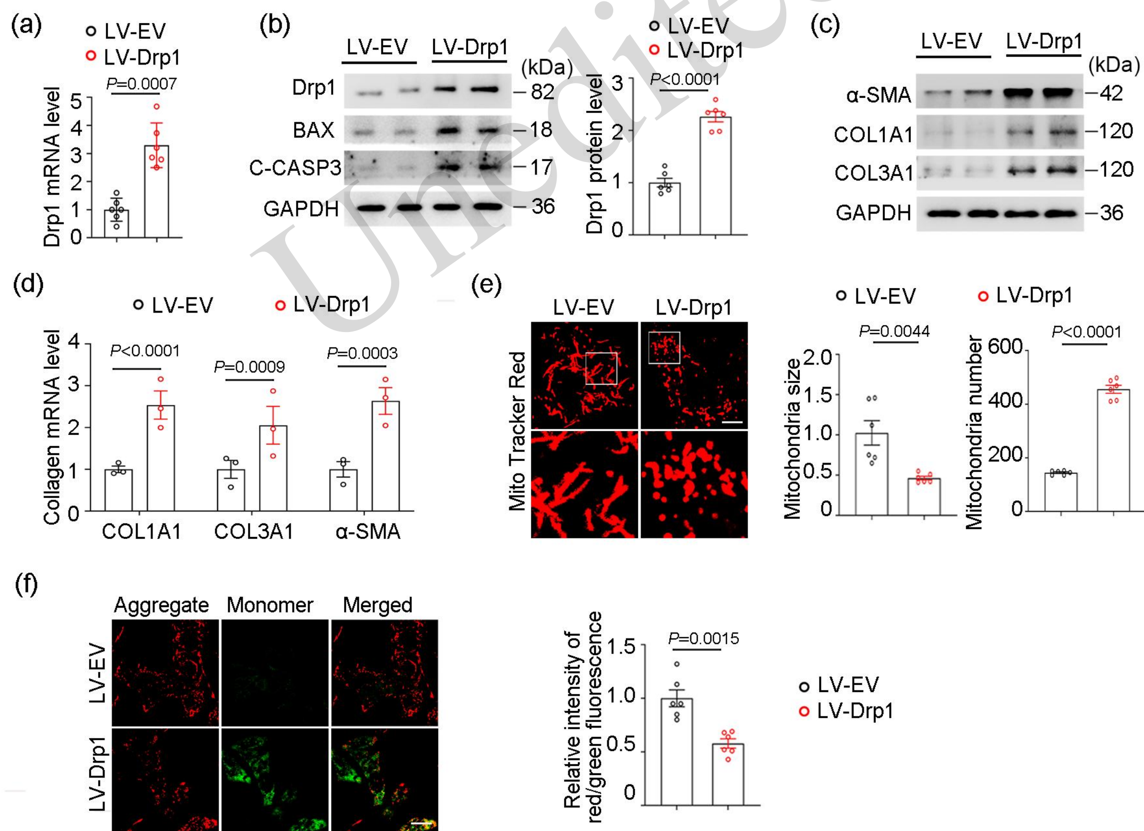

Fig. 3. Drp1 overexpression-mediated mitochondrial dysfunction and apoptosis in Dox-treated cardiomyocytes. (a-e) Primary cardiomyocytes were infected with lentivirus encoding Drp1 (LV-Drp1) or lentivirus empty vector (LV-EV). (a) Cells were subjected to RT-qPCR analysis to examine the mRNA expression of Drp1 (n = 6). (b) Primary cardiomyocytes were infected with lentivirus LV-Drp1 or LV-EV, and then treated with Dox. Cells were collected for immunoblotting analysis, and the protein levels of Drp1, BAX and C-CASP3 were examined (n = 6). (c) Cardiac fibroblasts were infected with lentivirus LV-Drp1 or LV-EV,

and then treated with Dox. Cells were collected for immunoblotting (n = 6). (d) RT-qPCR analysis and the protein and mRNA level of fibrosis molecules were examined (n = 3). (e) Mitochondrial morphology was displayed by staining with MitoTracker Red, and the number and size of mitochondria were analyzed. Scale bar, 20 μm (n = 6). (f) The mitochondrial membrane potential was examined by staining with JC-1, and the relative intensity of red/green was measured. Scale bar, 20 μm (n = 6). Two-tailed unpaired Student's t test was used to compare the two groups (Figs. a-b and d-f).

2.4 Cardiac Drp1 depletion-mediated attenuation of Dox-induced cardiotoxicity in vivo

We further validated the protective effect of Drp1 deletion in Dox-induced cardiotoxicity in vivo by using cardiac-specific Drp1 knockout mice (*Drp1*$^{flox/flox}$–*Myh6*-Cre$^{+/+}$), hereafter referred to as conditional knockout (cKO) mice. Mice carrying floxed Drp1 alleles but lacking Cre recombinase expression (*Drp1*$^{flox/flox}$-*Myh6*-Cre$^{-/-}$) were used as controls, hereafter referred to as wild-type (WT) mice (Fig. S1b). The deletion of Drp1 significantly preserved cardiac function and mitigated cardiac injury following Dox exposure, as evidenced by the increased left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) (Figs. 4a and 4b). Morphological and histological analysis revealed that Drp1 deletion alleviated cardiac fibrosis induced by Dox (Figs. 4c and 4d). In addition, the depletion of Drp1 strikingly decreased the expression of apoptotic and fibrosis biomarkers in cardiac tissues upon Dox treatment (Figs. 4e-4g). Further analysis showed that the knockout of Drp1 reduced the excessive mitochondrial fission induced by Dox (Fig. 4h). The depletion of Drp1 in the heart restored aberrant mitochondrial membrane potential and mitochondrial ROS level induced by Dox treatment (Figs. S1c and S1d). These results demonstrate that Drp1 deletion in the heart reduced mitochondrial fission and exerted a protective role in Dox-induced cardiotoxicity.

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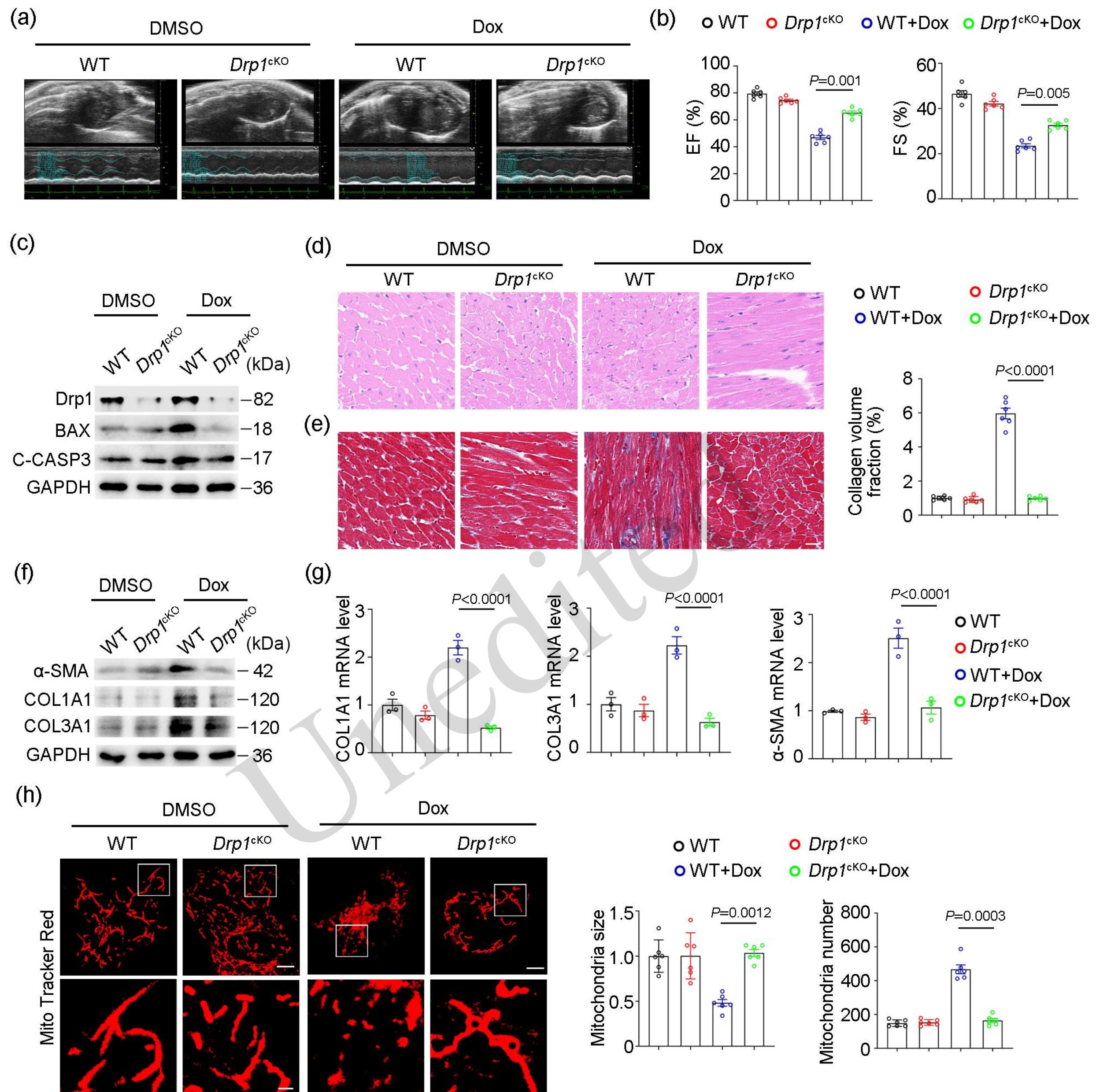

Fig. 4. Cardiac Drp1 depletion-mediated attenuation of Dox-induced cardiotoxicity in vivo. (a) Drp1 mice with cardiac specific deletion or WT mice were injected with Dox, and then subjected to echocardiography analysis (n = 6). (b) The left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) of mice hearts were calculated. (c) Cells were collected for immunoblotting analysis, and the protein level of Drp1, BAX and C-CASP3 were examined. (d and e) The HE staining and Masson's trichrome staining of the mice hearts were shown. Scale bar, 50 μm. The collagen volume fraction was measured (n = 6). (f) The protein expression of apoptosis and fibrosis markers in cardiac tissues were examined by immunoblotting. (g) The mRNA level of fibrosis markers in cardiac tissues were examined by RT-qPCR analysis (n = 3). (h) Primary cardiomyocytes were isolated from the mice hearts, then stained with MitoTracker Red, and the mitochondria number and size were analyzed. Scale bar, 20 μm (n = 6). One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. b, d and g-h).

2.5 Rhein-mediated rescue of Dox-induced cardiac damage in vivo

Research has indicated that rhein exerts a protective effect on the cardiovascular system (Chen, et al., 2024). We therefore examined the potential role of rhein in Dox-induced cardiotoxicity (Fig. 5a). Mice were first injected with Dox, then treated with rhein or Dex, an intracellular iron chelating agent exerting cardioprotective activity. Rhein treatment dramatically preserved cardiac function in a dose-dependent manner upon Dox exposure (Figs. 5b and 5c). Besides, morphological and histological analysis revealed that rhein treatment attenuated cardiac fibrosis in a dose-dependent manner induced by Dox (Figs. 5d and 5e). Furthermore, rhein significantly restored the expression level of fibrosis markers in mice hearts induced by Dox (Figs. 5f and 5g). These results indicate that rhein treatment restored the Dox-induced cardiac damage in vivo.

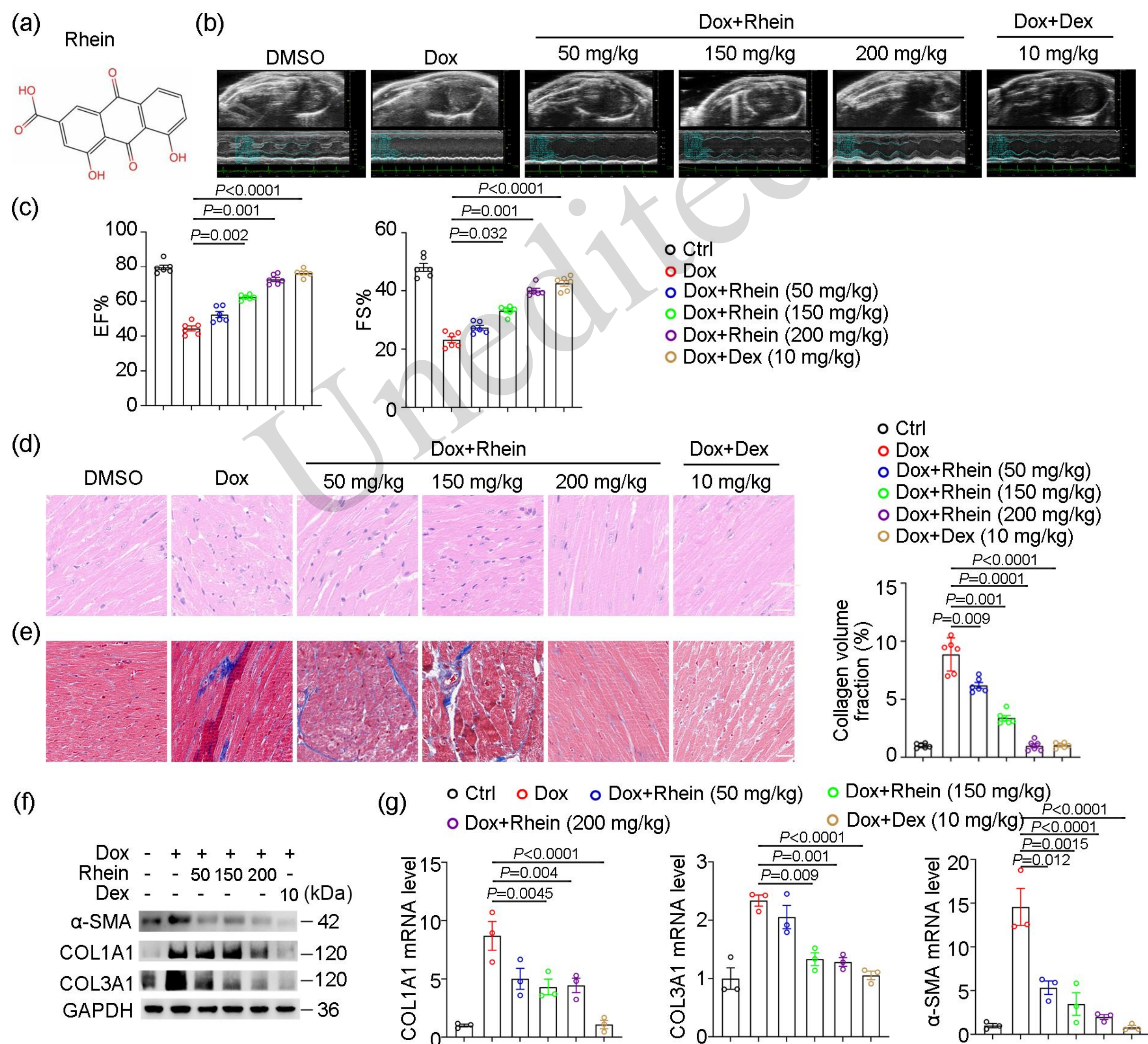

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Fig. 5. Rhein-mediated rescue of Dox-induced cardiac damage in vivo. (a) The formula of rhein is shown. (b) Mice were first injected with Dox, then treated with rhein or dexrazoxane (Dex) and subjected to echocardiography analysis (n = 6). (c) The left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) of mice hearts were measured. (d and e) The HE staining and Masson's trichrome staining of the

mice hearts were shown. Scale bar, 50 μm. The collagen volume fraction was measured. (f and g) The protein and mRNA level of fibrosis markers in cardiac tissues were examined by immunoblotting (f) and RT-qPCR (g) analysis (n = 3). One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. c, e and g).

2.6 Rhein-mediated attenuation of Dox-induced mitochondrial fission and ROS in cardiomyocytes

Given that Drp1-mediated mitochondrial fission was promoted upon Dox treatment, we next investigated whether rhein restored Dox-induced cardiac damage by regulating mitochondrial dynamics. We found that rhein attenuated the elevated expression of Drp1 induced by Dox treatment in a dose-dependent manner (Figs. 6a and 6b). Besides, rhein treatment attenuated the excessive mitochondrial fission induced by Dox (Fig. 6c). In addition, rhein significantly enhanced MMP and decreased mitochondrial ROS level upon Dox treatment (Figs. 6d and 6e). These data demonstrate that rhein defended against Dox-induced cardiotoxicity by attenuating Drp1-mediated mitochondrial fission and mitochondrial ROS production in cardiomyocytes.

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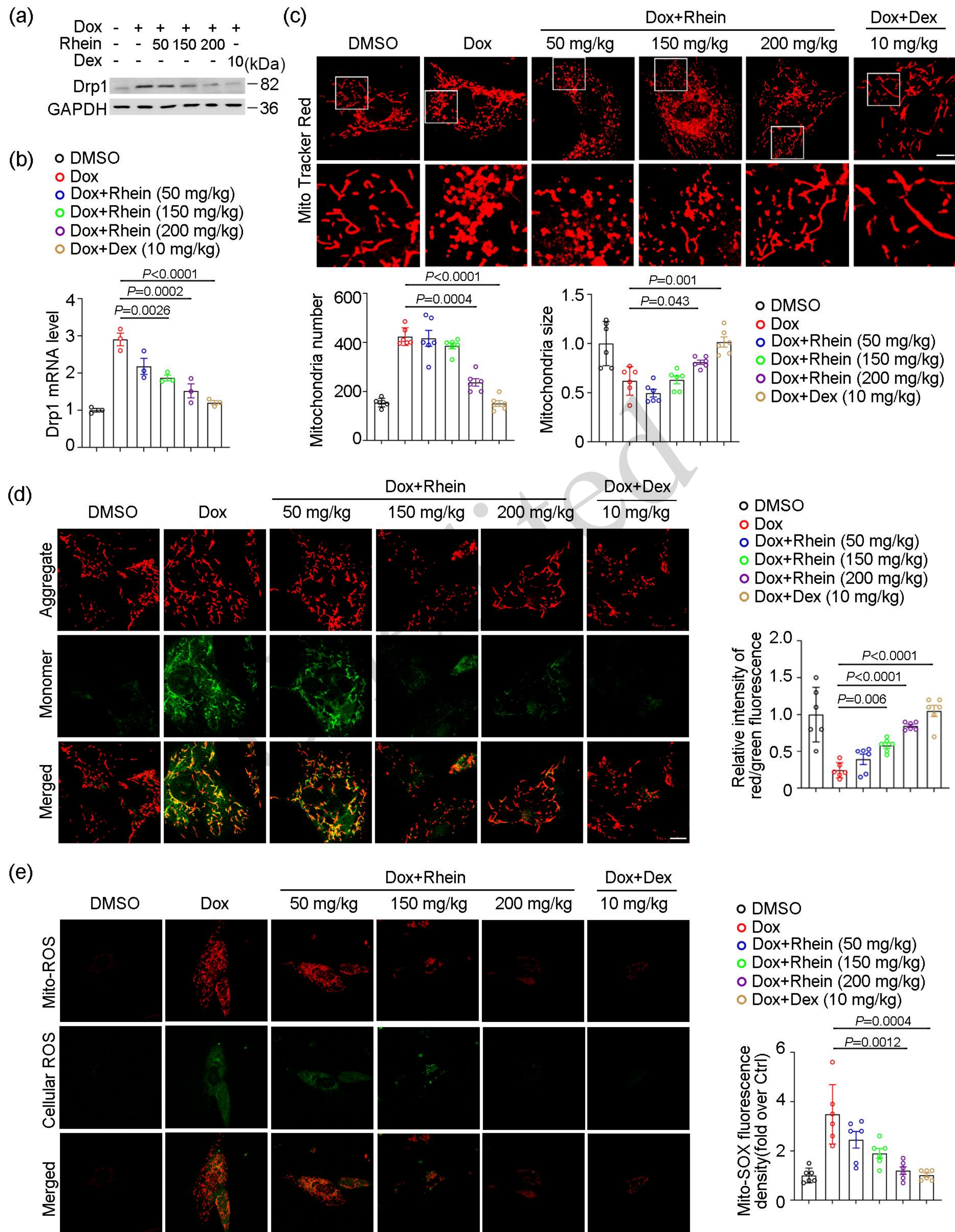

Fig. 6. Rhein-mediated attenuation of Dox-induced mitochondrial fission and ROS in cardiomyocytes. (a and b) Mice were first injected with Dox, then treated with rhein or Dex. The protein and mRNA levels of Drp1 in cardiac tissues were examined by immunoblotting (a) and RT-qPCR (b) analysis (n = 3). (c-e) Primary

cardiomyocytes were isolated from the mice hearts. Cells were stained with MitoTracker Red, and the mitochondria number and size were recorded. Scale bar, 20 μm (n = 6) (c). The mitochondrial membrane potential was examined by staining with JC-1 in cardiomyocytes, and the relative intensity of red/green was measured. Scale bar, 20 μm (n = 6) (d). The mitochondrial ROS and cellular ROS in cardiomyocytes were stained by MitoSOX and DCFH-DA, respectively. Scale bar, 20 μm (n = 6) (e). One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. b-e).

2.7 Rhein-mediated attenuation of Dox-induced Drp1 upregulation via NR3C1 translocation inhibition

We conducted further investigation into the molecular mechanisms involved underlying the cardioprotective role of rhein by regulating mitochondrial fission. A comprehensive analysis of Animal TFDBv4.0, PROMO and JASPAR databases yielded three overlapping potential transcription factors regulating Drp1 upon Dox treatment, including NR3C1, vitamin d receptor (VDR) and RXRA (Fig. 7a). To identify the specific transcription factor that regulates Drp1, we treated primary cardiomyocytes with Dox and rhein or Dex. Among these transcription factors, rhein treatment only inhibited the nuclear translocation of NR3C1 using histone H3 (H3) as a nuclear marker (Fig. 7b). Consistently, gene expression profiling analysis of RNA-seq data (GSE157904) from murine hearts revealed that Dox treatment significantly upregulated the expression of NR3C1 (Fig. 7c). Besides, the protein and mRNA expression levels of NR3C1 were dramatically enhanced in primary cardiomyocytes treated with Dox (Figs. 7d and 7e). Considering the existence of Dox-induced Drp1-mediated mitochondrial fission, we then investigated whether NR3C1 is involved in this process. We found that the knockdown of NR3C1 strikingly abolished the elevated expression of Drp1 (Figs. 7f and 7g), as well as inhibited excessive mitochondrial fission upon Dox treatment (Fig. 7h). These results demonstrate that rhein attenuated the upregulation of Drp1 via inhibiting the nuclear translocation of NR3C1 upon Dox exposure.

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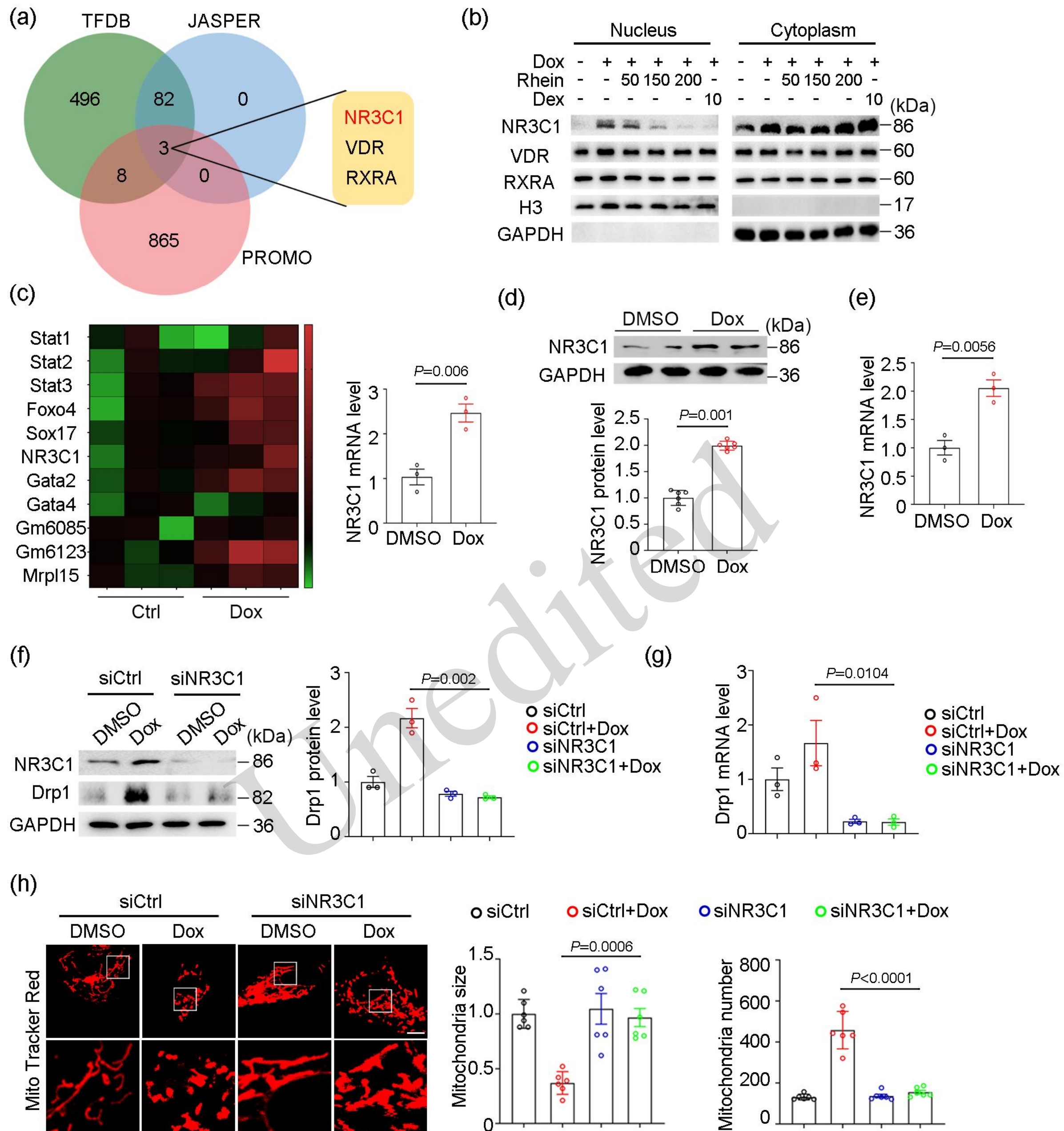

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Fig. 7. Rhein-mediated attenuation of Dox-induced Drp1 upregulation via nuclear receptor subfamily 3, group c, member 1 (NR3C1) translocation inhibition. (a) Venn diagram showing 3 overlapping potential transcription factors regulating Drp1 upon Dox treatment. (b) Primary cardiomyocytes were treated with Dox and rhein, or Dex, and the cytoplasmic and nuclear lysates were separated after cell harvesting. The lysates were subjected to immunoblotting. (c) Comparative gene expression profiling analysis of RNA-seq data (GSE157904) for hearts in mice treated with DMSO or Dox. The mRNA level of NR3C1 was analyzed ($n = 3$). (d and e) Primary cardiomyocytes were treated with Dox, and the protein ($n = 6$) (d) and mRNA ($n = 3$) (e) levels of NR3C1 were measured. (f and g) Primary cardiomyocytes were transfected with control or NR3C1 siRNA, and then treated with Dox for 24 h. The protein and mRNA level of Drp1 were examined by immunoblotting ($n = 3$). (h) Primary cardiomyocytes were transfected with control or NR3C1 siRNA, and then treated with Dox for 24 h. Mitochondrial morphology was demonstrated by staining with MitoTracker Red, and the number and size

of mitochondria were analyzed. Scale bar, 20 μm (n = 6). Two-tailed unpaired Student's t-test was used to compare the two groups (Figs. c and e). One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. f-h).

2.8 Rhein-mediated alleviation of Dox-induced Drp1 upregulation via PI3K/Akt pathway activation

Studies have shown that the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway inhibits cardiomyocyte apoptosis induced by Dox (Kitamura et al., 2014; Zhang et al., 2020). We therefore explored whether the PI3K/Akt signaling pathway is involved in the cardioprotective effects of rhein against Dox-induced cardiotoxicity by using MK-2206 (10 μM), a specific Akt pathway inhibitor. We found that MK-2206 treatment significantly abrogated the protective effect of rhein against Dox-induced cardiotoxicity in primary cardiomyocytes with Dox together with MK-2206 treatment, as shown by the restored expression of apoptotic protein BAX and C-CASP3, as well as the elevated level of Drp1 and NR3C1 (Fig. 8a). Forkhead box a1 (FOXA1) serves as a well-defined downstream transcriptional effector of the PI3K/Akt pathway, whose activity is tightly regulated by Akt-mediated phosphorylation (Du et al., 2017; Liu et al., 2017). Conversely, primary cardiomyocytes treated with Dox and rhein or a specific Akt activator SC79 significantly promoted the expression of phosphorylated Akt (P-Akt), phosphorylated phosphoinositide 3-kinase (P-PI3K) and FOXA1, indicating that rhein might attenuate Dox-induced cardiotoxicity via regulating PI3K/Akt signaling pathway (Fig. 8b). To further validate the role of FOXA1 in the cardioprotective role of rhein, primary cardiomyocytes were transfected with FOXA1 siRNA, and treated with Dox (3 μM, 24h) and rhein (60 μM, 24h). We found that rhein promoted the activation of PI3K/Akt and inhibited Drp1 expression, ultimately regulating mitochondrial fission in cardiomyocytes (Figs. 8a and 8b). The knockdown of FOXA1 abrogated the protective role of rhein against Dox-induced cardiotoxicity, as evidenced by increased mitochondrial fission, reduced MMP, and increased ROS (Figs. 8c-8f). Notably, FOXA1 knockdown abolished the inhibitory effect of rhein on NR3C1 nuclear translocation, indicated by the elevated phosphorylation of NR3C1 at Ser211, which was responsible for its nuclear translocation and subsequent transcriptional activation (Fig. 8f). These findings indicate that rhein suppressed the expression of NR3C1 and reduced its nuclear translocation by regulating the expression and activity of FOXA1. Thus, rhein promoted the activation of PI3K/Akt, which in turn modulated the expression and activity of FOXA1 to suppress the transcription of NR3C1, thereby inhibiting Drp1 expression and ultimately attenuating Dox-induced cardiotoxicity.

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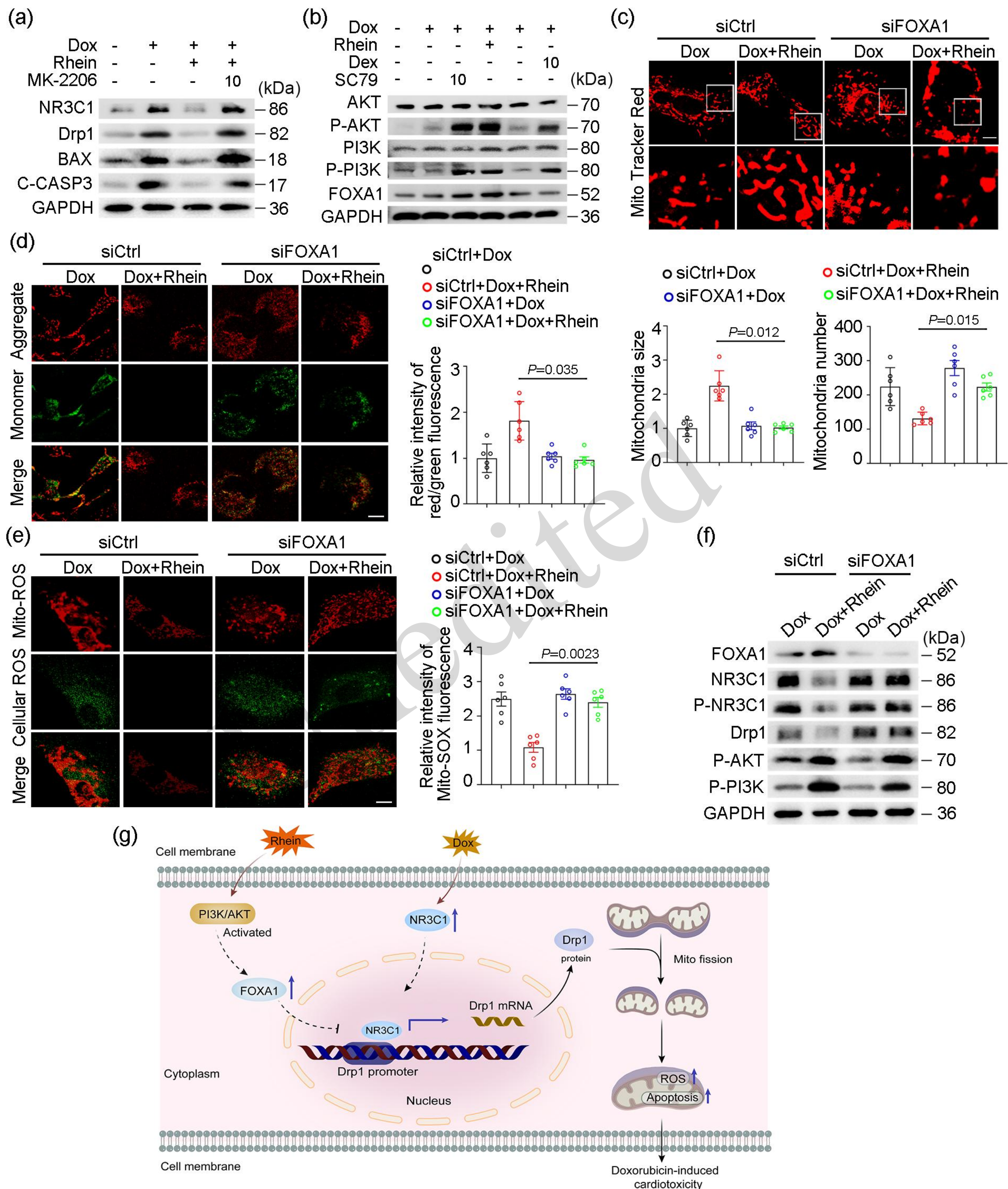

Fig. 8. Rhein-mediated alleviation of Dox-induced Drp1 upregulation via PI3K/Akt pathway activation. (a) Primary cardiomyocytes were treated with Dox, rhein or MK-2206, and then subjected to immunoblotting analysis. (b) Primary cardiomyocytes were treated with Dox together with rhein, or Akt signaling inhibitor MK-2206, or activator SC79 and then subjected to immunoblotting analysis. (c-e) Primary cardiomyocytes were transfected with FOXA1 siRNA, and then treated with Dox or together with rhein. Mitochondrial morphology was demonstrated by staining with MitoTracker Red, and the number and size of mitochondria

were analyzed. Scale bar, 20 μm (*n* = 6) (c). The mitochondrial membrane potential was examined by staining with JC-1 in cardiomyocytes, and the relative intensity of red/green was measured (*n* = 6). Scale bar, 20 μm (d). The mitochondrial ROS and cellular ROS in cardiomyocytes were stained by MitoSOX and DCFH-DA, respectively. Scale bar, 20 μm (*n* = 6) (e). (f) Primary cardiomyocytes were transfected with control or FOXA1 siRNA. Cells were collected and subjected to immunoblotting analysis to examine the protein level. (g) Working model. Dox treatment promoted mitochondrial fission and elevated the expression of NR3C1 in cardiomyocytes. The upregulation of NR3C1 promoted the transcriptional expression of Drp1 directly by binding to its promoter and thus induces mitochondrial fission. Further investigation showed that rhein attenuates Dox-induced cardiotoxicity by inhibiting NR3C1 nuclear translocation and regulating Drp1-mediated mitochondrial fission through the PI3K/Akt pathway. One-way ANOVA with Tukey's multiple comparison test was used to compare the groups (Figs. c-f).

3 Discussion

A growing body of evidence has highlighted that Dox-based anticancer therapy may induce functional and structural impairments in the heart, significantly limiting its clinical application. To date, effective therapeutic strategies for Dox-induced cardiotoxicity remain limited. Mitochondria serve as signaling hubs in the cell by regulating energy production, cell death, and buffering intracellular calcium (García-Peña et al., 2024). Mitochondrial dynamics, including mitochondrial fusion and fission, represent an important homeostatic mechanism for mitochondrial quality control (García-Peña, et al., 2024). Alterations in mitochondrial dynamics may contribute to increased mitochondrial ROS and impaired mitochondrial function, which can ultimately facilitate the progression of cardiovascular diseases (García-Peña, et al., 2024). Reports indicate that ischemia causes excessive mitochondrial fission and fragmentation, resulting in cardiomyocyte death (Chen, et al., 2023). Dox triggers Drp1 activation primarily by promoting the phosphorylation of Drp1 at Ser616, a well-established activating site, while concurrently reducing its inhibitory phosphorylation at Ser637 (Zeng et al., 2020; Yue et al., 2025). These two post-translational modifications coordinately enhance Drp1 translocation to the mitochondrial outer membrane and contribute to excessive mitochondrial fission. In this study, we found that Dox treatment promoted mitochondrial fission, and the suppression of Drp1-mediated mitochondrial fission was beneficial for Dox-induced cardiac injury. Our finding is consistent with the report that suppressing the excessive mitochondrial fission has a protective role for cardiovascular diseases (Ding, et al., 2018; Jin, et al., 2021).

Studies have increasingly demonstrated the protective role of rhein in cardiovascular diseases (Barbosa, et al., 2020; Li, et al., 2022). It has been reported that rhein protects myocardial cells from hypoxia/reoxygenation injury by blocking glycogen synthase kinase 3β (GSK3β) activity (Chen, et al., 2024). In addition, argirein, a derivative of rhein was effective in reversing the reduction of FKBP12.6/12 expression in cardiomyocytes treated with isoproterenol (Chen, et al., 2024). However, whether rhein attenuates cardiovascular diseases by regulating mitochondrial dynamics remains unclear. An early study reported that rhein protects pancreatic β-cells from Drp1-mediated mitochondrial fission and cell apoptosis under hyperglycemia (Liu et al., 2013). Rhein exerted cardioprotective effects against transverse aortic constriction-induced pathological cardiac hypertrophy, fibrosis and myocardial damage both in vivo and in vitro, and this protective effect is mechanistically mediated by the downregulation of the abnormal activation of p38 MAPK and STAT3 signaling pathways (Li, et al., 2022). Our study provides the first evidence that rhein alleviates Dox-induced cardiotoxicity by reducing Drp1-mediated mitochondrial fission via inhibiting the nuclear translocation of NR3C1 and mitochondrial ROS level in cardiomyocytes. Besides mitochondrial fission, mitochondrial fusion is vital for maintaining mitochondrial quality and function. It has been reported that Mfn2-mediated mitochondrial fusion mitigates Dox-induced cardiotoxicity (Ding et al., 2022). It will be intriguing to explore in further studies whether rhein attenuates cardiac injury by regulating mitochondrial fusion.

Abundant studies have documented that rhein

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exerts broad-spectrum anticancer activity against multiple malignancies, such as by inducing cell cycle arrest and apoptosis, inhibiting tumor progression and angiogenesis, reversing chemoresistance, and modulating key oncogenic PI3K/Akt/mTOR, NF-κB and MAPK pathways (Wu et al., 2017; Henamayee et al., 2020). The dosage of rhein (50 mg/kg) used in our study was close to the clinical tolerance dosage in humans, indicating a favorable clinical translation potential of rhein (Reagan-Shaw et al., 2008; Wojcikowski and Gobe, 2014). In a Phase II clinical trial for diabetic nephropathy, oral administration of rhein at 100-200 mg per day was tolerated well in subjects, with no severe adverse events such as abnormal hepatic or renal dysfunction. Furthermore, clinical studies have confirmed that daily oral doses of 150 mg for 4 weeks and 200 mg for 12 weeks did not cause dose-limiting toxicity, with only mild gastrointestinal reactions observed (Wu et al., 2020; Zeng et al., 2021). Accumulating preclinical evidence underscores that rhein does not interfere with the anticancer efficacy of Dox (Wu et al., 2019). Instead, it exerts synergistic antitumor effects and reverses Dox resistance in multiple cancer models via inhibiting mitochondrial energy metabolism, enhancing intracellular Dox accumulation, and promoting tumor cell apoptosis (Wu, et al., 2019; Wu, et al., 2020).

It is well-established that the PI3K/Akt signaling pathway plays a critical role in regulating the mechanisms of cardiomyocyte death, which may contribute to stress-induced cardiac dysfunction. Akt activation prevents Dox-induced cardiomyocyte apoptosis, whereas Akt inhibition exacerbates Dox-induced cardiomyocyte apoptosis and impaired cardiac function (Zhang, et al., 2020). COX5A exerted a protective role in Dox-induced cardiomyopathy by regulating PI3K/Akt signaling (Zhang et al., 2023). Consistently, our study found that rhein alleviates Dox-induced cardiotoxicity by activating the PI3K/Akt signaling pathway. The etiology of Dox-induced cardiotoxicity has been linked to several underlying mechanisms, such as mitochondrial ROS, mitochondrial dysfunction, impaired autophagy, ferroptosis, and apoptosis (Zhang, et al., 2023). Our study mainly explored the cardioprotective role of rhein in Dox-induced cardiotoxicity by mediating mitochondrial dynamics and function. However, it is also worthy to explore whether rhein attenuates cardiac damage by regulating autophagy or ferroptosis (Yao et al., 2025).

Our study provides evidence that the genetic depletion of FOXA1 results in a marked increase in NR3C1 phosphorylation at the Ser211 residue, a post-translational modification well-established to promote NR3C1 nuclear import and transcriptional competency (Wang et al., 2002; Rider et al., 2018). In line with this functional link between FOXA1 and NR3C1 signaling, the ablation of FOXA1 fully reverses the inhibitory effect of rhein on NR3C1 nuclear translocation. Taken together, our data support a working model wherein rhein exerts its negative regulation on NR3C1 signaling through the modulation of FOXA1 expression and bioactivity, which in turn diminishes NR3C1 expression and limits its nuclear accumulation and downstream transcriptional activation. The results indicate that rhein exerts a direct regulatory effect on Drp1-mediated mitochondrial fission, which is evidenced by its inhibition of Drp1 expression and its mitochondrial translocation, as well as the modulation of NR3C1-Drp1 transcriptional axis. Meanwhile, we acknowledge that the antioxidant and anti-apoptotic activities of rhein may act in a reciprocal and synergistic manner to reinforce mitochondrial homeostasis. The reduced mitochondrial ROS due to the antioxidant activity of rhein may further dampen Drp1 hyperactivation, and the preserved mitochondrial dynamics in turn alleviates mitochondrial ROS overproduction and apoptotic signaling, forming a positive feedback loop. While our data support that the PI3K/Akt/NR3C1/Drp1 cascade participates in rhein-mediated protection against Dox-induced cardiotoxicity, alternative pathways are likely involved in regulating NR3C1 or Drp1. One such example is the p38 MAPK-mediated site-specific phosphorylation of NR3C1, which bidirectionally regulates its nucleocytoplasmic shuttling and transcriptional activity independent of the PI3K/Akt cascade (Huang et al., 2025). Besides, the Hsp90-FKBPs chaperone complex orchestrates the ligand binding and nuclear translocation of NR3C1, which operates independently of Akt-mediated phosphorylation (Kaziales et al., 2020; Noddings et

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al., 2023). It will be necessary to explore the alternative signaling pathways regulating the NR3C1/Drp1 cascade in the cardioprotective role of rhein against Dox-induced cardiotoxicity.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

All data and materials are available from the corresponding author upon request

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Author contributions

Huan YUE: Conception, methodology; investigation; data curation. Runjing LI: Conception, validation; investigation. Jiajia XU: Conception, validation; investigation. Weixin LIU: Validation; investigation. Ziyang ZHAO:Validation; investigation. Junxiao FENG: Validation; investigation. Rui SHI: Validation; investigation. Dongkun XIE: Investigation. Zhenghao ZHANG: writing-review and editing; funding acquisition. Xingjuan SHI: Writing-original draft; writing-review and editing; funding acquisition.

Compliance with ethics guidelines

Huan YUE , Runjing LI, Jiajia XU, Weixin LIU, Ziyang ZHAO, Junxiao FENG, Rui SHI, Dongkun XIE, Zhenghao ZHANG and Xingjuan SHI declare that they have no conflicts of interest. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Southeast University (approval number: SEU-IACUC-20250227201).

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Supplementary information

Table 1; Fig. S1; Materials and methods

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