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Original article

CTRP3 protected against doxorubicin-induced cardiac dysfunction, inflammation and cell death via activation of Sirt1

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ABSTRACT

Background: Inflammation and myocytes apoptosis play critical roles in the development of doxorubicin (DOX)-induced cardiotoxicity. Our previous study found that C1q/tumour necrosis factor-related protein-3 (CTRP3) could inhibit cardiac inflammation and apoptosis of myocytes but its role in DOX-induced heart injury remains largely unknown. Our study aimed to investigate whether CTRP3 protected against DOX-induced heart injury and the underlying mechanism.

Methods: We overexpressed CTRP3 in the hearts using an adeno-associated virus system. The mice were subjected to a single intraperitoneal injection of DOX (15 mg/kg) to induce short-term model for cardiomyopathy. The morphological examination and biochemical analysis were used to evaluate the effects of CTRP3. H9C2 cells were used to verify the protective role of CTRP3 in vitro.

Results: Myocardial CTRP3 protein levels were reduced in DOX-treated mice. Cardiac specific-overexpression of CTRP3 preserved heart dysfunction, and attenuated cardiac inflammation and cell loss induced by DOX in vivo and in vitro. CTRP3 could activate silent information regulator 1 (Sirt1) in vivo and in vitro. Moreover, specific inhibitor of Sirt1 and the silence of Sirt1 could abolish the protective effects of CTRP3 against DOX-induced inflammation and apoptosis.

Conclusion: CTRP3 protected against DOX-induced heart injury via activation of Sirt1. CTRP3 has therapeutic potential for the treatment of DOX cardiotoxicity.

1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most effective chemotherapeutic agents. It has been widely applied as the first-line drugs to treat human neoplasms, including leukemias, lymphomas and solid malignancies [1]. However, the clinical use of DOX is limited due to undesirable side effects. DOX-induced cardiotoxicity could lead to irreversible degenerative cardiomyopathy and congestive heart failure [2]. The pathogenesis of cardiotoxicity induced by DOX remains poorly understood, but emerging data implicate an indispensable role for inflammation and apoptosis [3,4].

It has been proved that DOX could increase the production of proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which activated apoptosis-related signaling pathway and eventually resulted in the loss of cardiomyocytes [5–8]. Notably, the activation of nuclear factor-κB (NF-κB) is essential for the development of DOX-mediated cardiotoxicity and inhibition of NF-κB could attenuate DOX-induced cardiotoxicity [9,10]. Silent information regulator 1 (Sirt1) has been shown to inhibit NF-κB pathway [11]. Moreover, activating Sirt1 by resveratrol prevented DOX-related cardiotoxicity via mitochondrial stabilization [12]. Therefore, it is important to find a positive regulator of Sirt1 during DOX-induced cardiotoxicity.

C1q/tumour necrosis factor-related protein-3 (CTRP3), a novel adipokine, is a member of the highly conserved CTRP family of adiponectin paralogs [13,14]. It has been demonstrated that CTRP3 acts as a novel and endogenous antagonist of lipopolysaccharide [15]. CTRP3 has been reported to reduce apoptosis and prevent cardiac fibrosis in the ischemic mouse heart [16,17]. Our previous study found that CTRP3 was also highly expressed in the heart reaching approximately half that in adipose tissue. Moreover, knockdown of CTRP3 in cardiomyocytes resulted in inflammation and cell apoptosis at baseline and

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supplement of CTRP3 improved cardiac function in diabetic rats [18]. As another adipokine, CTRP9 induced mitochondrial biogenesis via activating Sirt1 pathway [19]. However, the hypothesis whether CTRP3 could protect against DOX-induced cardiotoxicity via Sirt1 still remains unverified. In the present study, we report our investigation into the protective effects of CTRP3 in DOX-induced cardiac injury.

2. Materials and methods

2.1. Reagents

DOX (D1515, purity ≥ 98%) and EX-527 (E7034, purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were purchased from Cell Signaling Technology (Boston, MA, USA): CTRP3 (1:1000), Caspase3 (1:1000), C-Caspase3 (1:1000), p-NF-κB (1:1000), NF-κB (1:1000), BAX (1:1000), BCL-2 (1:1000), Sirt1 (1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000). Proliferating cell nuclear antigen (PCNA, 1:200) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-rabbit/mouse EnVisionTM + /HRP reagent was purchased from Gene Technology (Shanghai, China), and Alexa Fluor 488- and 568-goat anti-rabbit secondary antibody was purchased from LI-COR Biosciences (Lincoln, USA). The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). The cell counting kit-8 was obtained from Dōjindo Laboratories (Kumamoto, Japan). Adeno-associated virus (AAV) carrying CTRP3 or Green fluorescent protein (GFP) used in our study was previously generated by Hanbio (Shanghai, China) [18].

2.2. Animals and treatments

All animal care and experimental procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, which is also under the guidance of Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, revised 2011). All the animal treatments and subsequent analysis were performed in a blind fashion for all groups. Male C57BL/6 mice (8- to 10-week-old; body weight: 25.5 ± 2 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). The source of AMPKα2 knockout mice was described in our previous article [20]. These mice were allowed free access to food and water under a 12 h light-dark cycle and housed with controlled temperature (20–25 °C) and humidity (50 ± 5%) in the Cardiovascular Research Institute of Wuhan University (Wuhan, China). C57BL/6 mice were divided into four groups, normal saline (NS) + GFP, NS + CTRP3, DOX + GFP and DOX + CTRP3, by a random number table. The mice were then given single injection of 5×10^{10} viral genome particles/mouse (AAV-CTRP3 or AAV9-GFP, diluted in 15 μL PBS) via tail vein. Four weeks after the injection, mice in DOX group were subjected to a single intraperitoneal injection of DOX (15 mg/kg) to induce short-term model for cardiomyopathy while the control animals were given equal volume of saline [21]. These mice were observed daily and weighed every two days. Eight days later, these mice were euthanized with an overdose of sodium pentobarbital (200 mg/kg; i.p.). To induce a chronic model of DOX-induced cardiotoxicity, the mice were injected intraperitoneally with DOX (5 mg/kg, once a week, the total cumulative dose is 15 mg/kg) for 3 times beginning at 4 weeks after AAV-GFP or AAV-CTRP3 injection [22]. Six weeks after DOX treatment, cardiotoxicity were evaluated. In reversed experiments, mice were treated with a specific inhibitor of Sirt1 (EX-527, 1 mg/kg) every other day for total 8 days [23]. To investigate whether AMPKα was involved in protection of CTRP3 against DOX-induced cardiac injury, AMPKα2 knockout mice were subjected to DOX (15 mg/kg) at 4 weeks post the injection of AAV-CTRP3. At the endpoint of treatment, all the mice were anesthetized with 1.5% isoflurane, echocardiographic measurements and hemodynamic analysis were performed. After that, hearts were weighed and snap-frozen in liquid nitrogen for the further detection.

2.3. Echocardiography and hemodynamics

After the mice were anesthetized using isoflurane (1.5%), echocardiography was performed by a MyLab 30CV ultrasound (Esaote SpA, Genoa, Italy) with a 10-MHz linear array ultrasound transducer. M-mode images of the left ventricle at the papillary muscle level were recorded and then left ventricular internal diastolic diameter (LVIDd) and the fractional shortening (FS) were measured [18,20,24–27].

Hemodynamic variables were analyzed using a Millar catheter transducer (SPR-839; Millar Instruments, Houston, TX). The data were analyzed using the PVAN data analysis software.

2.4. Immunohistochemistry staining

The removed hearts were fixed in 10% formalin overnight. The hearts were transversely sectioned into 5 μm slices. Then the slices were stained with haematoxylin and eosin (HE). Immunohistochemistry staining of TNF-α and CD68 were performed to detect the myocardial inflammation. The sections were incubated with anti-TNF-α (Abcam, 1:100) or anti-CD68 (Abcam, 1:100) at 4 °C overnight and followed by EnVisionTM + /HRP reagent incubation at 37 °C for 1 h. After that, diaminobenzidin (DAB) was used to visualize the sections for 2 min at room temperature. After mounting by neutral gums, these sections were determined by the light microscopy (Nikon (Tokyo, Japan), H550L). The sections were examined blind.

2.5. Western blot and quantitative real-time PCR

The total proteins were extracted from the frozen heart tissues or iced cell lysates by RIPA agent (Invitrogen, Carlsbad, CA, USA). Nuclear protein extracts were isolated using commercial kits. The proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (cat. Number IPFL00010; EMD Millipore, Billerica, MA, USA). Then the membranes were blocked with 5% nonfat milk at room temperature and incubated with the primary antibodies at 4 °C overnight. After incubating with the secondary antibodies at 37 °C for 1 h, the PVDF membrane were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Total RNAs were isolated and reverse-transcribed to cDNA by Transcriptor First Strand cDNA Synthesis Kit [Roche (Basel, Switzerland), 04896866001]. The quantification of real time PCR were performed using the LightCycler 480 SYBR Green Master Mix (cat. Number 04896866001; Roche). All primer details were provided in Table S1. The total protein levels were normalized to GAPDH and nuclear protein were normalized to PCNA. Phosphorylation was normalized to the matched total protein. The mRNA data were normalized to *Gapdh*.

2.6. Cell culture

H9C2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, C11995), containing 10% fetal bovine serum (FBS, GIBCO, 10099), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO, 15140), at 37 °C in a humidified atmosphere with 5% CO_2. Then, H9C2 cells were divided into four groups: the PBS group, the PBS + CTRP3 group, the DOX group and the DOX + CTRP3 group. CTRP3 was purchased from Aviscera Bioscience (Santa Clara, CA, USA), which has been previously described [18]. The cells were seeded onto 6-well in DMEM with 10% FBS for 48 h. After that, the cells were starved for 16 h and then were treated with DOX (1 μmol/L) or DOX + CTRP3 (3 μg/mL) for 12 h. To detect cell viability, the cells were seeded onto 96-well in DMEM with 10% FBS for

48 h, after being starved, these cells were with DOX (1 μmol/L) or DOX + CTRP3 (3 μg/mL) for 24 h. Cell viability was detected by cell counting kit-8 (CCK-8).

2.7. siRNA transfection

siRNAs targeting Sirt1 were generated by RiboBio (RiboBio Co., Ltd., Guangzhou, China). The cells were transfected with siSirt1 or a scrambled RNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three siRNA were generated and the one resulted in the most downregulation of Sirt1 protein level were used for the further study.

2.8. Immunofluorescence and TUNEL staining

The immunofluorescence and TUNEL Staining were performed as previously described [19,23]. Briefly, the sections or cell coverslips were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. After that, the sections or cell coverslips were incubated with anti-Caspase3 (1:100) at 37 °C for 2 h and the following secondary antibody (1:100). The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining was performed according to the manufacturer's instructions using a commercially available kit (Millipore, USA) to detect apoptosis. After that, the sections were mounted with DAPI, and were observed under the OLYMPUS DX51 fluorescence microscope (Tokyo, Japan). All the of images were quantified by Image-Pro Plus 6.0.

2.9. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The differences between two groups were performed with unpaired Student's *t*-test. The differences were analyzed with one-way analysis of variance (ANOVA) with Tukey post-hoc analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CTRP3 improved cardiac function of mice with DOX treatment

To investigate whether CTRP3 is involved in DOX-induced cardiotoxicity, we initially sought to determine the expression of CTRP3 in ventricular samples of mice with acute DOX treatment. Immunofluorescence staining and western analysis revealed that myocardial CTRP3 protein levels were reduced in DOX-treated mice (Fig. 1a–b). Therefore, we overexpressed CTRP3 in the hearts and found that cardiac-specific overexpression of CTRP3 could attenuate body weight loss induced by DOX injection (Fig. 1c–d). DOX injection resulted in the decreased the ratio of heart weight (HW) and tibia length (TL), however, there was no difference between DOX and DOX + CTRP3 group (Fig. 1e). Meanwhile, our results showed a decline in heart rate and maximum pressure blood in DOX group compared with mice in the control group at 8 days after DOX treatment, and mice with CTRP3 overexpression had unchanged heart rate and maximum pressure blood compared with mice treated with DOX (Fig. S1a–b). The echocardiography results revealed a decreased shortening fraction (FS) in DOX-treated mice, and conversely CTRP3 overexpression could improve mice FS (Fig. 1f). Echo data were listed in Supplement Table S2. Mice with DOX treatment for 8 days led to a significant decline in maximal rate of the increase of left ventricular pressure (+dP/dt) and the maximal rate of the decrease of left ventricular pressure (−dP/dt), after supplement of CTRP3, an increase in +dP/dt and −dP/dt was evident (Fig. 1g–h).

3.2. CTRP3 suppressed inflammation responses in acute DOX-treated mice

To observe the morphological change, HE staining was performed. As shown in Fig. 2a, DOX treatment resulted in the accumulation of inflammatory cells, and conversely CTRP3 overexpression significantly reduced the numbers of inflammatory cells. Moreover, overexpression of CTRP3 in the hearts also inhibited the increase of CD68-positive macrophages and the protein level of TNF-α induced by DOX treatment (Fig. 2a–c). Subsequent analysis of mRNA expression levels of *Tnf-α*, *Il-1β*, *Il-6* and *Mcp-1* at 8 days after DOX treatment further demonstrated that myocardial inflammation was suppressed by CTRP3 (Fig. 2d). NF-κB plays a key role in DOX-induced cardiac injury [28]. Next, we detected the alteration of NF-κB. DOX resulted in translocation of NF-κB from cytoplasm to nucleus, and this pathological change was attenuated by overexpression of CTRP3 in the hearts (Fig. 2e). To further confirm this change, nuclear protein was isolated, and we found that nuclear NF-κB was significantly increased after the treatment of DOX, which was inhibited by CTRP3 overexpression (Fig. 2f–g).

3.3. Overexpression of CTRP3 exhibited anti-apoptosis effect in DOX-induced cell death

As shown in Fig.3a, 8 days post DOX treatment, an increased level of apoptosis was observed in DOX-treated mice and CTRP3 overexpression could decrease TUNEL-positive cells (Fig. 3a). The anti-apoptosis effects of CTRP3 were further confirmed by western blot results showing that CTRP3 upregulated B-cell lymphoma 2 (BCL-2) and downregulated C-Caspase3. However, there was no change of BAX protein expression level between DOX group and DOX + CTRP3 group (Fig. 3b–c). Immunostaining revealed that CTRP3 attenuated the protein level of Caspase3 in the heart of mice with DOX treatment (Fig. 3d).

3.4. CTRP3 could protect hearts against DOX-induced chronic toxicity in vivo

Next, we investigated whether CTRP3 could attenuate DOX-induced chronic cardiotoxicity. As shown in Fig. 4a–c, repeated injection of DOX with a total cumulative dose of 15 mg/kg resulted in the decreased ratio of HW/TL, +dP/dt and FS after 6 weeks from the last injection, however, CTRP3 overexpression attenuated these pathological changes. (Echo data were listed in Supplement Table S3). CTRP3 also significantly decreased the mRNA levels of *Anp* and *Bnp* in DOX-treated hearts (Fig. 4d–e). To clarify that the similar mechanism is still working in chronic model, we detected the alteration of myocardial inflammation and cardiomyocytes apoptosis. The translocation of NF-κB from cytoplasm to nucleus was inhibited by the overexpression of CTRP3 (Fig. 4f–g). The decreased markers of inflammation further revealed the protective role of CTRP3 in DOX-induced chronic toxicity (Fig. 4h). We also found that CTRP3 reduced TUNEL-positive cells in the hearts of mice with chronic DOX treatment (Fig. 4i).

3.5. CTRP3 inhibited DOX-induced inflammation and cell loss via activating Sirt1 in H9C2 cells

Our previous study demonstrated that CTRP3 attenuated diabetes-related cardiomyopathy via activating AMPKα. Therefore, we first investigated whether CTRP3 protected against DOX-induced cardiotoxicity via AMPKα pathway. Unexpectedly, CTRP3 still alleviated DOX-caused cardiac injury even in mice with AMPKα deficiency (Fig. S2a–b). Moreover, previous studies have demonstrated that Sirt1 could suppress NF-κB, thus regulating cardiomyocyte inflammation and apoptosis [29,30], therefore we determined whether CTRP3 activated Sirt1 in vivo and in vitro. As shown in Fig.5a, Sirt1 protein expression was reduced by DOX and was restored after overexpression of CTRP3 in the hearts. Consistent with our finding in vivo, the protein level of Sirt1 was significantly increased in H9C2 cells treated with CTRP3 (Fig. 5b).

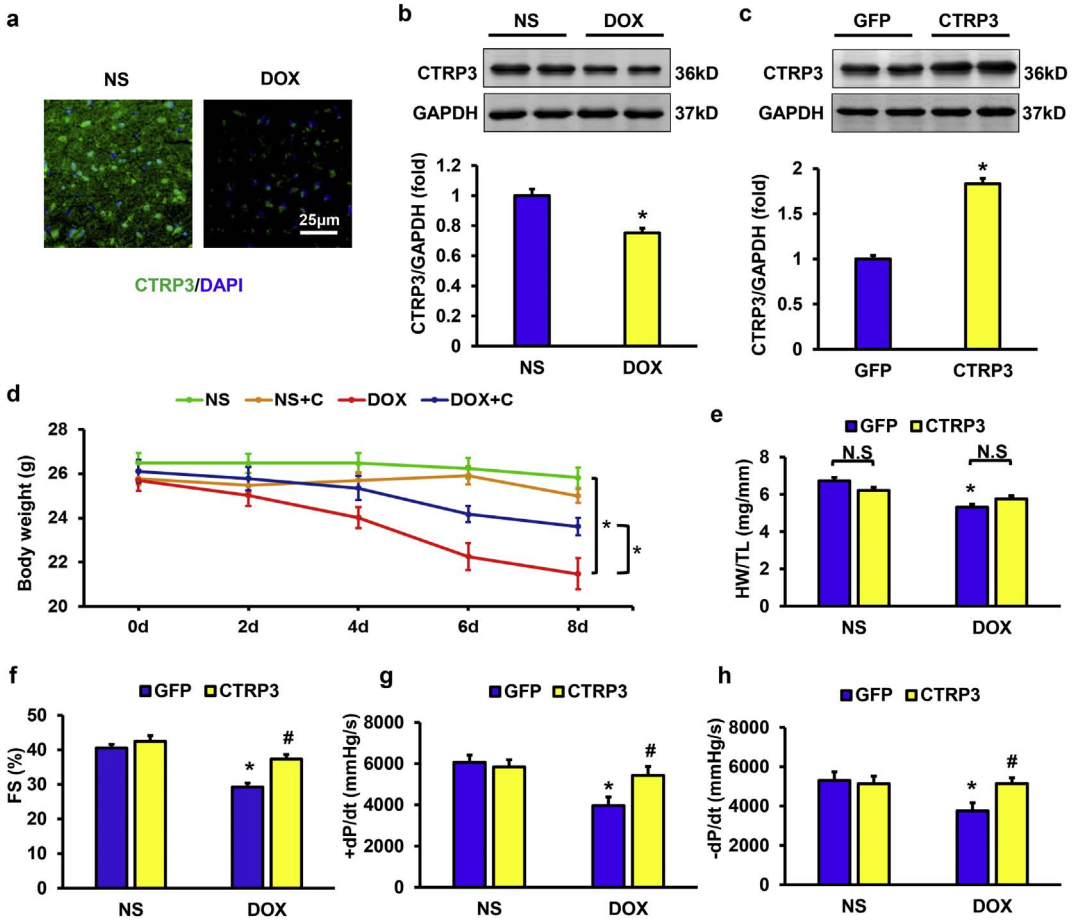

Fig. 1. C1q/tumour necrosis factor-related protein-3 (CTRP3) improved cardiac function in mice with doxorubicin (DOX) treatment. (a) Eight days after the injection of DOX (15 mg/kg), immunofluorescence of CTRP3 in normal saline (NS) and DOX-treated mice at (n = 4) at 8 days post the injection of DOX. (b) Representative western blot analysis of CTRP3 in the hearts (n = 6). (c) Four weeks after the injection of AAV-CTRP3 or AAV-GFP, the protein level of CTRP3 (n = 6). (d) Body weight of four groups (n = 9–12). (e) Statistical results of the heart weight (HW)/tibia length (TL) (n = 9–12). N·S means no significant difference. (f) Fraction shortening (FS) of mice after overexpression of CTRP3 (n = 9–12). (g–h) Hemodynamic analysis of mice with or without CTRP3 treatment (n = 7–11). Values represent the mean ± SEM. $^{*}P < 0.05$ versus NS + GFP, $^{\#}P < 0.05$ versus DOX + GFP. For figure 1b, $^{*}P < 0.05$ versus NS. For figure 1c, $^{*}P < 0.05$ versus GFP.

Next, we further investigated the effects of CTRP3 in vitro. H9C2 cells were transfected with siSirt1. The nuclear accumulation of NF-κB was significantly decreased after CTRP3 treatment, and the protection of CTRP3 was abolished by the knockdown of *Sirt1* (Fig. S3a, Fig. 5c). The subsequent detection of the mRNA levels of *Tnf-α* and *Il-1β* revealed that CTRP3 treatment inhibited DOX-induced inflammation in H9C2 cells (Fig. 5d–e), and CTRP3 lost anti-inflammatory effect after *Sirt1* deficiency. CTRP3 decreased DOX-induced cell loss in vitro, as indicated by TUNEL staining and apoptosis-related genes detection, and conversely knockdown of *Sirt1* in H9C2 cells almost completely offset anti-apoptosis effect of CTRP3 (Fig. 5f–i).

3.6. Sirt1 inhibition abolished the protective effects of CTRP3 in vivo

To further validate the mechanism, we injected mice with EX-527, the inhibitor of Sirt1. The intraperitoneal injection of EX-527 resulted in the decreased level of Sirt1 (Fig. 6a). EX-527 abolished CTRP3-induced protection, as shown by the decreased FS, + dP/dt and − dP/dt (Fig. 6b–d). The anti-inflammation effects of CTRP3 were blocked after the treatment of EX-527, as indicated by the increased NF-κB nuclear translocation and mRNA levels of *Tnf-α* and *Il-1β* (Fig. 6e–h). Moreover, the subsequent detection of the protein levels of C-Caspase3 and BCL-2 revealed that Sirt1 deficiency abolished anti-apoptosis effect of CTRP3 (Fig. 6i–j).

4. Discussion

In this study, we for the first time demonstrated DOX decreased the protein level of CTRP3 and cardiac-specific overexpression of CTRP3 could attenuate DOX-induced cardiac dysfunction by dramatically downregulating inflammation responses and apoptosis of cardiomyocytes in vivo and in vitro. Moreover, we found these protective effects were mediated by the activation of Sirt1 in vivo and in vitro, and knockdown or inhibition of Sirt1 abolished CTRP3-mediciated protection. Our study provided a novel approach for the treatment of DOX-induced cardiac injury.

Inflammation has been proved to be one of the critical processes of cardiac injury induced by DOX. DOX activated NF-κB, thus promoting the release of inflammatory cytokines and ultimately leading to cardiac dysfunction [28,31]. Our previous research has demonstrated that knockdown of CTRP3 resulted in the increased mRNA level of *Tnf-α*, meanwhile, overexpression of CTRP3 led to the decreased mRNA level of *Tnf-α* at baseline [18]. Therefore, we investigated whether CTRP3 could inhibited DOX-induced cardiac inflammation and found that C-TRP3 could suppress the nuclear translocation of NF-κB, thus decreasing the mRNA level of *Tnf-α*, *Il-1β*, *Il-6* and *Mcp-1*. It's well-evident that Sirt1 could suppress the expression of NF-κB [32]. Another question raised concerns the pathway through which CTRP3 exerted its anti-inflammatory effects. Depletion of Sirt1 by genetic approach or the specific inhibitor completely abolished CTRP3-mediated cardioprotection, implying a key role of Sirt1 in anti-inflammatory effect of CTRP3.

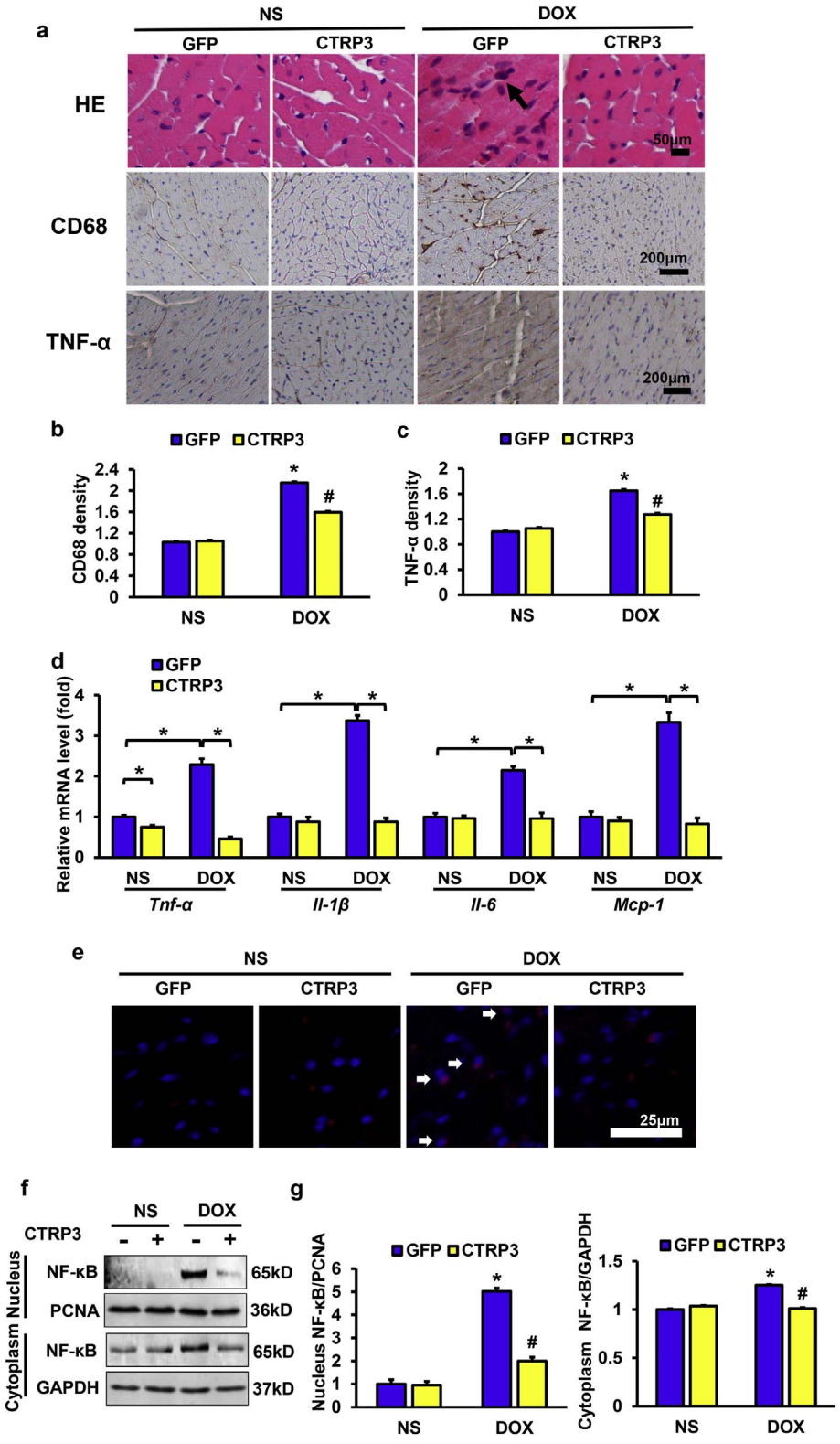

Fig. 2. C1q/tumour necrosis factor-related protein-3 (CTRP3) suppressed inflammation responses in doxorubicin (DOX)-treated mice. (a) HE staining and representative images of TNF-α and CD68 + macrophages in hearts with or without CTRP3. Arrows indicate inflammatory cells (n = 4). (b–c) Statistical results of CD68 + macrophages and TNF-α (n = 4). (d) PCR analysis of inflammation-related genes (n = 6). (e) Representative images of p-NF-κB immunofluorescence in mice with or without CTRP3 treatment (n = 4). Arrows indicate the nuclear translocation of p-NF-κB. (f–g) Western blot and quantitative results (n = 6). Values represent the mean ± SEM. $^{*}P < 0.05$ versus NS + GFP, $^{\#}P < 0.05$ versus DOX + GFP. In figure 2d, $^{*}P < 0.05$.

Inflammation results in the cell apoptosis. Previous studies demonstrated that CTRP3 attenuated cardiomyocyte apoptosis in the ischemic mouse hearts [1,13,33,34]. Our previous research also found that CTRP3 protected against cell death in diabetic hearts [18]. Consistent with these reports, we demonstrated CTRP3 inhibited DOX-induced cell loss in vivo and in vitro. The finding that Sirt1 deficiency abrogated CTRP3-mediated protection against apoptosis reveals a key role of Sirt1.

Sirt1 is a member of the class III group of histone deacetylases, and could be activated in response to various cellular stresses [35,36]. Accumulating evidence has indicated a role of Sirt1 in DOX-induced heart injury. Zhang et al. found Sirt1 protein level was slightly increased after DOX treatment [37]. However, inconsistent with this study, Danz et al. found that DOX induced a significant decrease Sirt1 activation, as measured by a decrease in acetylated histone H3 [38]. In line with this study, we also found that Sirt1 protein level was decreased after DOX

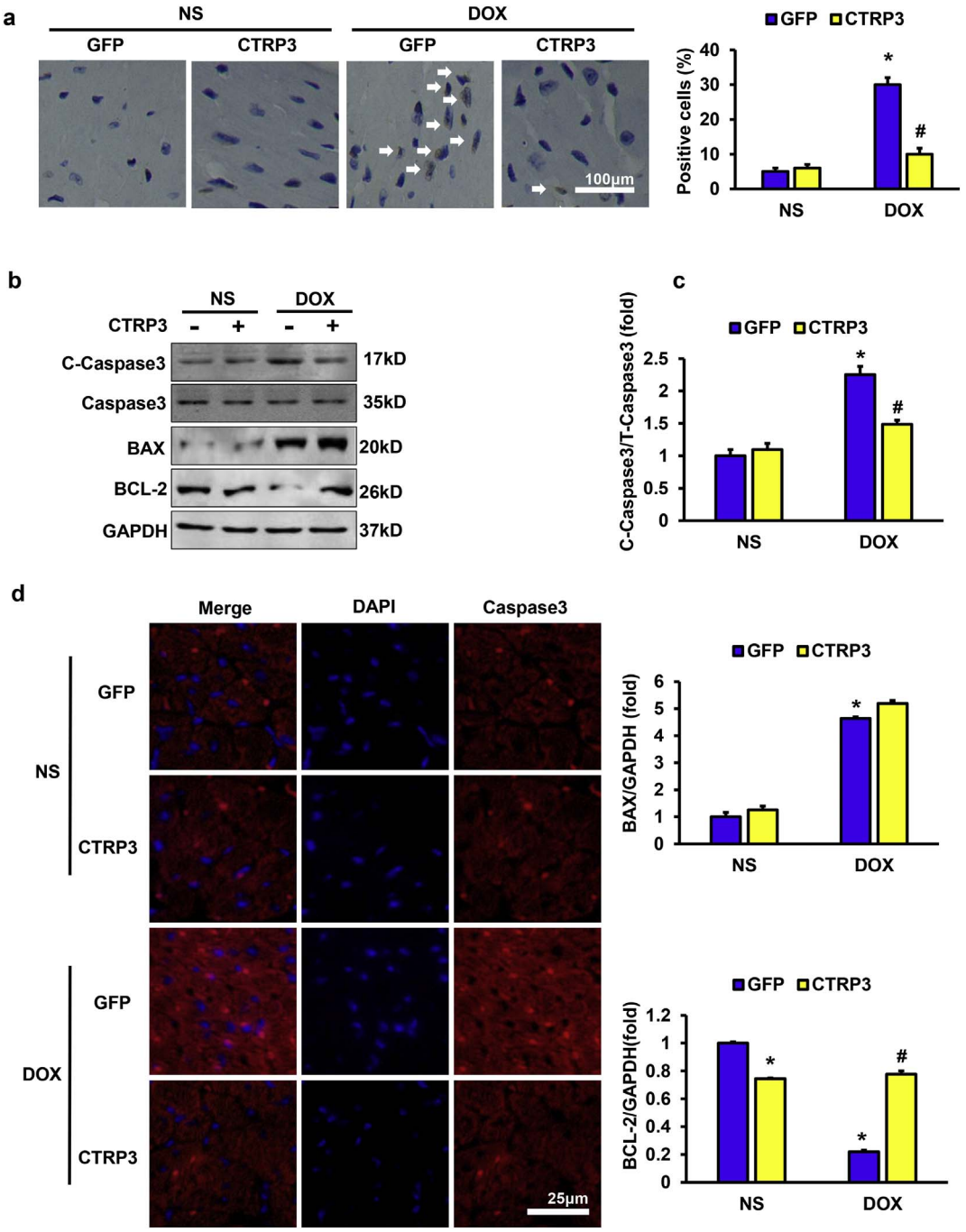

Fig. 3. Overexpression of C1q/tumour necrosis factor-related protein-3 (CTRP3) attenuated doxorubicin (DOX)-induced cell death in vivo. (a) Representative images of TUNEL and the quantitative results (n = 4). Arrows indicate TUNEL-positive cells. (b–c) Western blot and quantitative results in the indicated groups (n = 6). (d) Caspase3 immunofluorescence (n = 4). Values represent the mean ± SEM. $^{*}P < 0.05$ versus NS + GFP, $^{\#}P < 0.05$ versus DOX + GFP.

treatment in vivo and in vitro. Moreover, activating Sirt1 by CTRP3 in vivo and in vitro could attenuate DOX-induced cardiac dysfunction and knockdown of Sirt1 abolished the protective effects provided by CTRP3 in DOX-induced cardiotoxicity, indicating improved cardiac function caused by CTRP3 was at least partly mediated by Sirt1.

As a novel adipokine, little is known about CTRP3-related signaling pathway. Wu et al. found that CTRP3 attenuated cardiac fibrosis by inhibition nuclear translocation of Smad3 [16]. Yi et al. found that C-TRP3 induced significant protein kinase B (AKT) phosphorylation and thus activated HIF1α/VEGF pathway [13]. Hou et al. found that CTRP3 promoted proliferation of prostate cells through protein kinase C (PKC) signaling pathways [39]. Inconsistent with these findings, our previous study demonstrated that CTRP3 attenuated diabetes-related cardiac injury via AMPK pathway, which was in agreement with another report that CTRP3 promoted mitochondrial biogenesis through AMPK/proliferators activated receptor-γ co-activator-1α (PGC-1α) pathway [34]. More importantly, it has been reported that the activation of AMPK protected against various cardiac pathophysiological processes like inflammation and apoptosis [40–42]. These observations raised our hypothesis that CTRP3 exerted cardioprotection via AMPKα. Beyond our expectation, in our study, we found CTRP3 inhibited cardiac injury induced by DOX independent of AMPKα. Conversely, Sirt1 deficiency completely abolished the protection of CTRP3, implying that CTRP3 could suppress DOX-induced cardiotoxicity via activating Sirt1. These

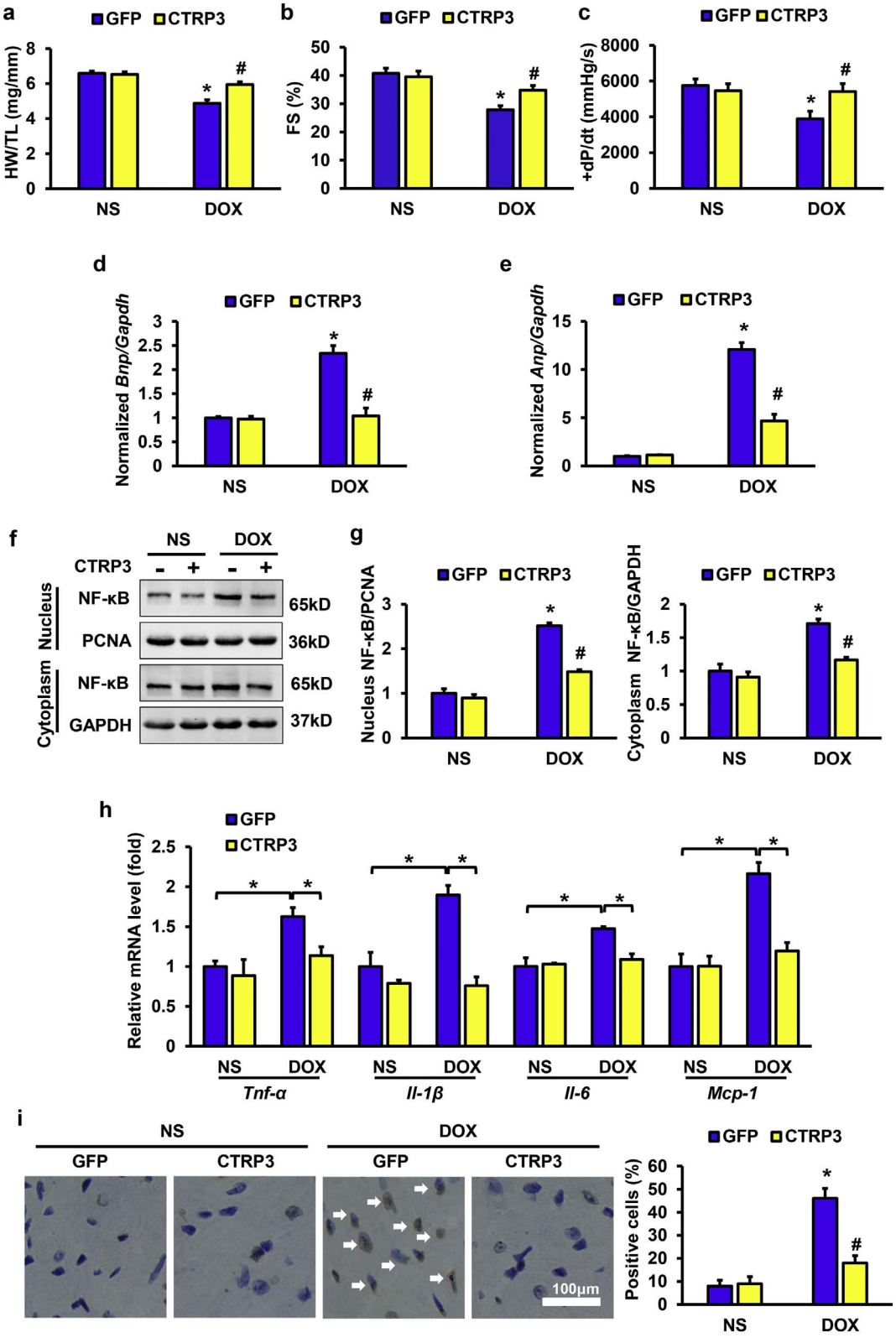

Fig. 4. C1q/tumour necrosis factor-related protein-3 (CTRP3) could protect hearts against doxorubicin (DOX)-induced chronic toxicity in vivo. (a) Statistical results of the heart weight (HW)/tibia length (TL) in mice treated with CTRP3 (n = 9–12). (b) Fraction shortening (FS) of mice at 6-week post the injection of DOX (n = 9–12). (c) Hemodynamic analysis of indicated groups (n = 9–12). (d) The mRNA level of *Bnp* (n = 6). (e) The mRNA level of *Anp* (n = 6). (f–g) Western blot and quantitative results (n = 6). (h) PCR analysis of inflammation-related genes (n = 6). (i) Representative images of TUNEL and the quantitative results (n = 4). Arrows indicate TUNEL-positive cells. Values represent the mean ± SEM. In figure 4h, $^{*}P < 0.05$. In others, $^{*}P < 0.05$ versus NS + GFP, $^{\#}P < 0.05$ versus DOX + GFP.

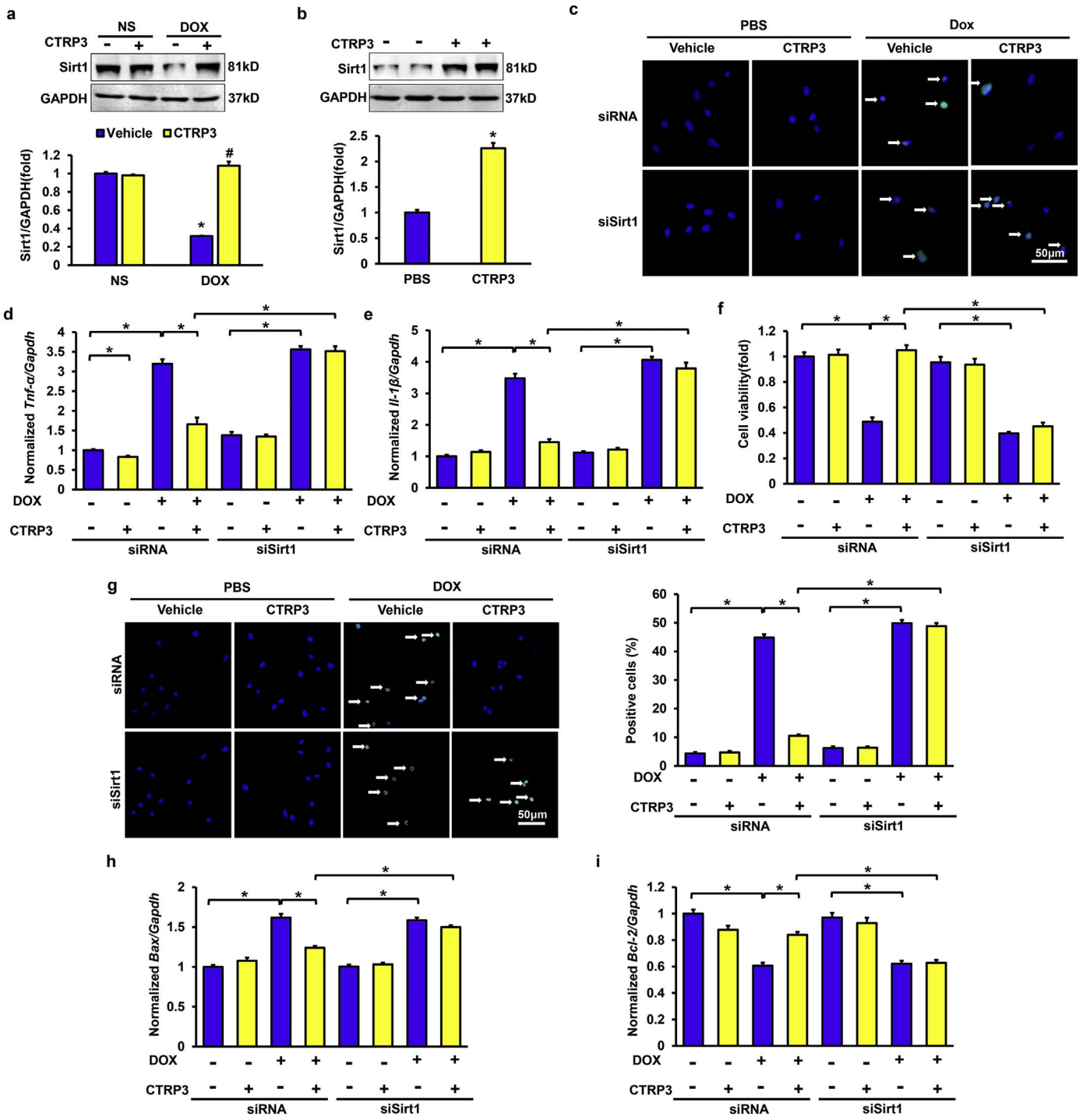

Fig. 5. C1q/tumour necrosis factor-related protein-3 (CTRP3) inhibited doxorubicin (DOX)-induced inflammation and cell loss via activating Silent information regulator 1 (Sirt1). (a) Western blot analysis of Sirt1 (n = 6). (b) Sirt1 expression level after supplement of CTRP3 in H9C2 (n = 3). (c) Representative images of p-NF-κB immunofluorescence in H9C2 cells with Sirt1 deficiency (n = 3). (d–e) The mRNA levels of inflammation-related genes (n = 3). (f) Cell viability after the treatment of DOX and CTRP3 (n = 3). (g) TUNEL staining and the quantitative results (n = 3). Arrows indicate TUNEL-positive cells. (h–i) The mRNA levels of apoptosis-related genes. Values represent the mean ± SEM from three independent experiments. In figure 5a, $^{*}P < 0.05$ versus NS + vehicle, $^{\#}P < 0.05$ versus DOX + vehicle. In figure 5b, $^{*}P < 0.05$ versus PBS group. In figure 5d–i, $^{*}P < 0.05$.

findings revealed the complexity of signaling pathways regulated by CTRP3, and further study will advance our understanding into the precise mechanism through which CTRP3 exerted its biological effects.

Collectively, the present study demonstrated that CTRP3 inhibited inflammation and apoptosis of cardiomyocytes, improved cardiac function after DOX treatment. Gene-based therapy targeting CTRP3 may be a promising therapeutic approach to treat chemotherapeutic agents-induced cardiotoxicity.

Conflicts of interest

None.

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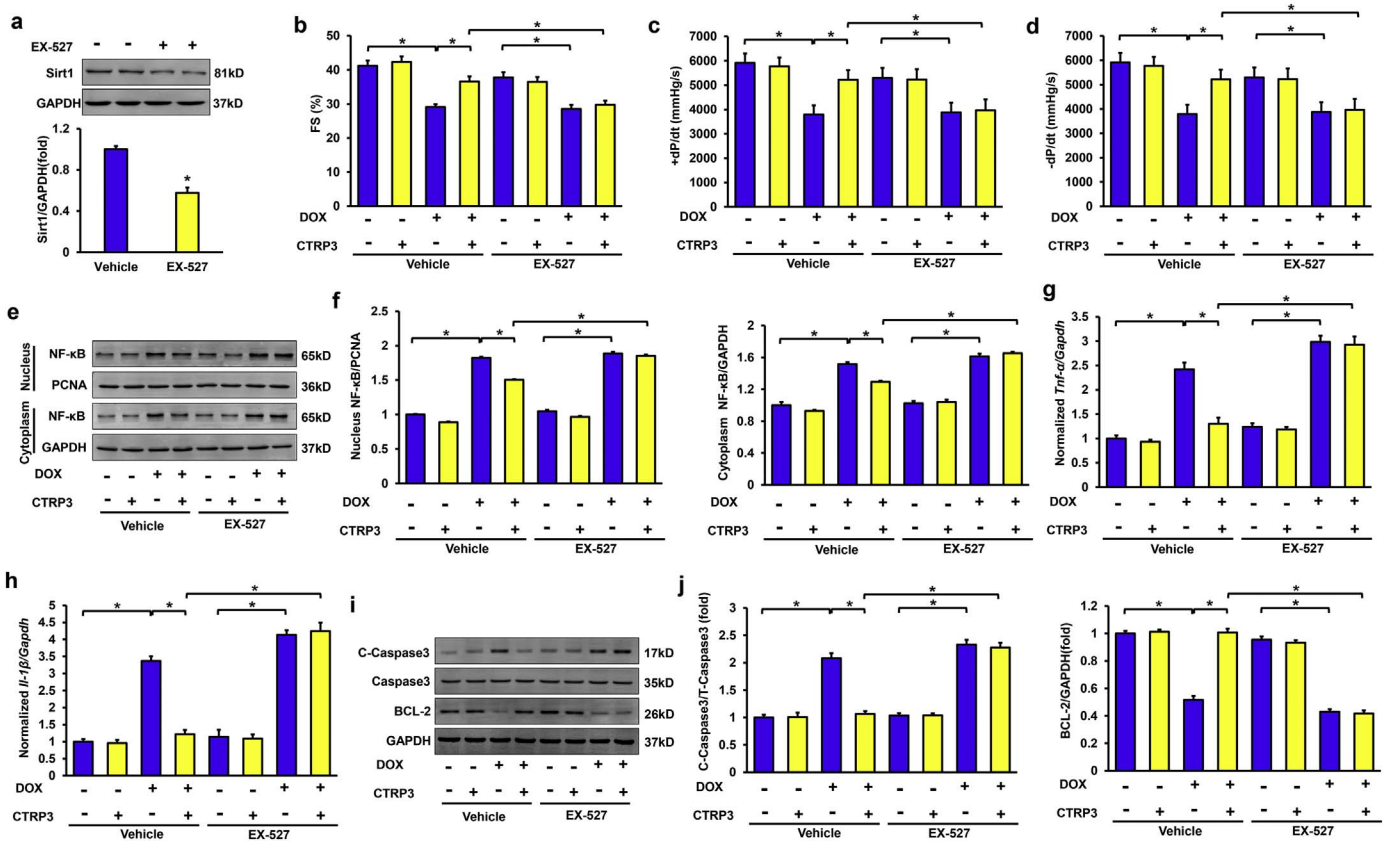

Fig. 6. Silent information regulator 1 (Sirt1) inhibition abolished the protective effects of CTRP3 in vivo. (a) Sirt1 expression level (n = 6). (b) Fraction shortening (FS) of mice after the inhibition of Sirt1 (n = 6). (c–d) Hemodynamic analysis of indicated groups (n = 6). (e–f) Western blot and quantitative results (n = 6). (g–h) The mRNA levels of *Tnf-α* and *Il-1β* (n = 6). Values represent the mean ± SEM. In figure 6a, $^{*}P < 0.05$ versus vehicle. In others, $^{*}P < 0.05$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2017.10.008.

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