Cathepsin B aggravated doxorubicin-induced myocardial injury via NF-κB signalling

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Abstract. Myocyte apoptosis and oxidative stress key critical roles in the process of doxorubicin (DOX)-induced cardiotoxicity. However, how apoptosis and oxidative stress arise in DOX-induced heart injury remains largely unknown. Cathepsin B (CTSB) is a typical lysosomal cysteine protease that is associated with apoptosis, inflammatory responses, oxidative stress and autophagy. The present study aimed to investigate the role of CTSB in DOX-induced heart injury and its potential mechanism. H9C2 cells were infected with adenovirus or transfected with small interfering RNA to overexpress or knock down CTSB, respectively, and then stimulated with DOX. DOX induced increased CTSB expression levels in H9C2 cells. DOX-induced cardiomyocyte apoptosis and oxidative stress were attenuated by CTSB knockdown but aggravated by CTSB overexpression *in vitro*. Mechanistically, the present study showed that CTSB activated the NF-κB pathway in response to DOX. In summary, CTSB aggravated DOX-induced H9C2 cell apoptosis and oxidative stress via NF-κB signalling. CTSB constitutes a potential therapeutic target for the treatment of DOX-induced cardiotoxicity.

Introduction

As a broad-spectrum anthracycline antitumour drug, doxorubicin (DOX) is widely used in chemotherapy to treat numerous types of tumour including solid tumours, transplantable leukaemia and lymphomas (1). However, the clinical application of DOX is limited by its cardiotoxicity, which manifests as congestive heart failure (2). Cardiomyocyte apoptosis, necrosis and other modes of cell death may be primary mechanisms underlying DOX-induced deterioration of cardiac function (3). In addition, excessive oxidative stress, lipid peroxidation, DNA damage and autophagy are also involved in this pathological process (4). Nevertheless, the progression and mechanisms underlying this process are still unclear. Investigating the molecular mechanism of DOX may help to identify a suitable strategy for the prevention and treatment of DOX-induced myocardial injury.

As a member of the papain family, Cathepsin B (CTSB) is a widely expressed lysosomal cysteine endopeptidase (5). High levels of CTSB are found in macrophages, osteoclasts and different types of cancer cells, including lung, colon, prostate, breast and gastric cancer (6). Moreover, CTSB is also expressed in cardiomyocytes, and increased CTSB expression levels and activity in the myocardium are reported to be induced by DOX (7,8), angiotensin II (9) and isoproterenol (10) and in patients with dilated cardiomyopathy (11). Additionally, CTSB is associated with apoptosis (12,13) and oxidative stress (14,15), which serve key roles in the process of DOX-induced myocardial injury. Our previous study demonstrated that CTSB was upregulated in the heart following pressure overload, and functions as a modulator of the hypertrophic response via regulating the TNF-α/apoptosis signal-regulating kinase 1 (ASK1)/JNK pathway (9). However, the mechanism by which CTSB regulates DOX-induced cardiotoxicity remains unverified. The present study demonstrated that CTSB exacerbated cardiomyocyte apoptosis and oxidative stress induced by DOX, and that the underlying mechanism was due to the activation of NF-κB signalling.

Materials and methods

Cell culture. H9C2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS at 37˚C in a humidified atmosphere with 5% CO_2. After culturing for 24 h, the cells were treated with 1 μM DOX or PBS. The specific NF-κB inhibitor JSH-23 (10 μmol/l) (16) was administered to the H9C2 cells to inhibit NF-κB activation for 24 h at 37˚C.

Cell counting kit (CCK)-8 assay. H9C2 cell viability was determined via CCK-8 assay according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, the

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cells were seeded in 96-well plates. Different concentrations (0.25, 0.50, 1.00, 2.50 or 5.00 μM) of DOX were used to treat the H9C2 cells for 24 h at 37˚C. Then, 10 μl CCK-8 was added to each well and incubated at 37˚C for 1 h. The optical density values were obtained at 450 nm.

Cell transfection. In order to overexpress CTSB, H9C2 cells were plated in 6-well plates and transfected with adenovirus (Ad)-CTSB (MOI, 100) or Ad-negative control (NC) for 6 h and then stimulated with 1 μM DOX or PBS for 24 h at 37˚C. In order to knock down CTSB expression levels, H9C2 cells were transfected with small interfering (si)RNA targeting CTSB (siCTSB; 50 nM) or scrambled siRNA (50 nM) for 24 h using 1X riboFECT™ CP Reagent according to the manufacturer's protocol. and then stimulated with 1 μM DOX or PBS for 24 h. The siCTSB sequence was 5'-GGACGACATGAT TAACTAT-3'. The siNC sequence was 5'-TTCTCCGAACGT GTCACGTdTdT-3' (Guangzhou RiboBio Co., Ltd.).

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as previously described (9). Briefly, TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA. A Transcriptor First-Strand cDNA Synthesis kit (Roche Diagnostics) was used to reverse transcribe the total RNA into cDNA. PCR amplifications were quantified using a LightCycler 480 SYBR-Green 1 Master Mix (Roche Diagnostics). The thermocycling conditions for qPCR were as follows: 95˚C for 30 sec; 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 95˚C for 15 sec; 60˚C for 1 min; 95˚C for 15 sec. The results were normalized to GAPDH gene expression levels. Relative gene expression levels were calculated using the $2^{-\Delta\Delta cq}$ method (17). The following primers were used: GAPDH: Forward, 5'-GACATGCCGCCTGGA GAAAC-3' and reverse, 5'-AGCCCAGGATGCCCTTTA GT-3'; GP91: Forward, 5'-GACCATTGCAAGTGAACA CCC-3' and reverse, 5'-AAATGAAGTGGACTCCACGCG-3'; P67: Forward, 5'-CGAGGGAACCAGCTGATAGA-3' and reverse, 5'-CATAGGCACGCTGAGCTTCA-3'; glutathione peroxidase (GPx): Forward, 5'-GAGAATGGCAAGAATGAA GAG-3' and reverse, 5'-GAAGGTAAAGAGCGGGTGA-3'.

Western blot analysis. Proteins were extracted from H9C2 cells, and the concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.) as previously described (9). Protein samples (50 μg) were separated by 10% SDS-PAGE (Wuhan Servicebio Technology Co., Ltd.) and then transferred to PVDF Immobilon-P transfer membrane (EMD Millipore). The membrane was blocked with 5% skimmed milk in Tris-buffered saline Tween-20 (Sigma-Aldrich) for 1 h at room temperature and then incubated overnight at 4˚C with the indicated primary antibodies. Primary antibodies against Bax (cat. no. 2772; Cell Signaling Technology, Inc.), CTSB (cat. no. 31718; Cell Signaling Technology, Inc.), GAPDH (cat. no. 2118; Cell Signaling Technology, Inc.), caspase-3 (cat. no. 9662; Cell Signaling Technology, Inc.), cleaved (c)-caspase-3 (cat. no. 9661; Cell Signaling Technology, Inc.), superoxide dismutase (SOD)1 (cat. no. ab16831; Abcam), Bcl-2 (cat. no. ab196495; Abcam), SOD2 (cat. no. ab68155; Abcam), NF-κB p65 (cat. no. ab16502; Abcam), phosphorylated (p)-NF-κB p65 (cat. no. ab194726; Abcam), IκBα (cat. no. ab7217; Abcam), and p-IκBα (cat. no. ab133462; Abcam) were used for western blotting. The dilution of all primary antibodies was 1:1,000. Then the membrane was incubated with goat anti-rabbit IgG secondary antibody (1:10,000; cat. no. A21020; Abbkine Scientific Co., Ltd.) for 1 h at room temperature. The blots were visualised using ECL Plus (Wuhan Servicebio Technology Co., Ltd.) reagent and a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc.). Image Lab software 3.0 (Bio-Rad Laboratories, Inc.) was used to perform quantification.

Oxidative stress detection. Detection of reactive oxygen species (ROS) in the H9C2 cells of each group was performed using a ROS Assay kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, after treatment, DCFH-DA (1:1,000) was added to H9C2 cells cultured in serum-free medium for 30 min in the dark at 37˚C, then cells were washed with PBS three times and observed under a fluorescence microscope at x200 magnification. Dihydroethidium (DHE; Beyotime Institute of Biotechnology) was used to detect the superoxide anion levels in cells. Briefly, 2 μM DHE was added to the H9C2 cells after treatment, and the cells were incubated at 37˚C for 30 min, then washed with PBS for three times.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (9). Briefly, following transfection with Ad-CTSB/Ad-NC or siCTSB/siNC and stimulation with DOX, the cells were fixed, permeabilized and blocked. Then, cells were incubated with primary antibodies against cathepsin B and p-NF-κB p65 at 4˚C overnight. The next day, the cells were washed with PBS three times and incubated with Alexa Fluor 488-conjugated goat anti-rat IgG (1:200; cat. no. A21090; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. Then, the cells were observed with a fluorescence microscope at x400 magnification.

Apoptosis assessment. TUNEL staining was performed according to the manufacturer's instructions with a ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit (EMD Millipore), Briefly, Cells were fixed in 1% paraformaldehyde for 10 min at room temp and post-fixed with precooled ethanol and Acetic acid (2:1) for 5 min at -20˚C. Next, the equilibration buffer was added for ~10 sec at room temperature, followed by 55 μl/5 cm^2 of working Strength TdT enzyme for 1 h at 37˚C. The specimens were then placed in a coplin jar containing stop/wash buffer, agitated for 15 sec and incubated for 10 min at room temperature. Then mounting medium containing 0.5-1.0 μg/ml propidium iodide was added for 1 min at room temperature and the images were captured (>50 fields per coverslip) via fluorescence microscopy at x200 magnification.

Statistical analysis. SPSS software (version 23.0; IBM Corp.) was used for analysis. The results are expressed as the mean ± SD of three independent repeats. Differences between two groups were analysed via the Student's t-test. One-way ANOVA was used to evaluate differences between multiple groups, followed by post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

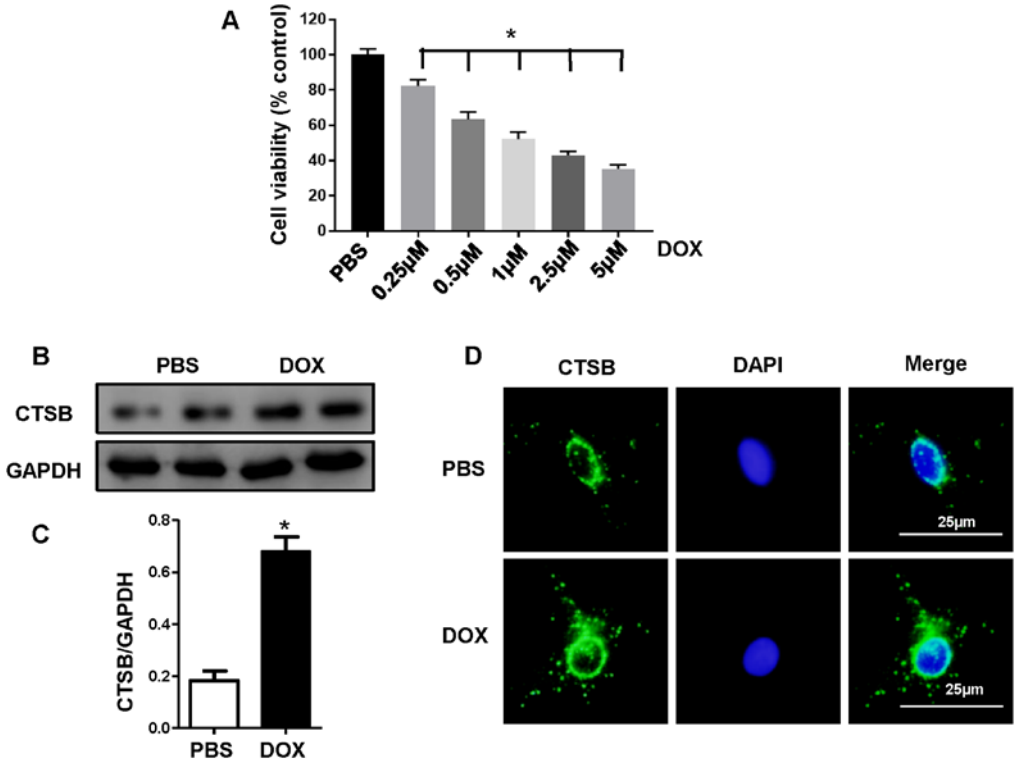

Figure 1. CTSB is upregulated in H9C2 cells treated with DOX. (A) Effect of DOX (0.25-5.00 μM) on cell viability. (B) Western blotting and (C) quantitative analysis of CTSB in H9C2 cells treated with DOX. (D) Immunofluorescence staining of CTSB in H9C2 cells stimulated with DOX or PBS. n=6. *P<0.05 vs. PBS. CTSB, cathepsin B; DOX, doxorubicin.

Results

CTSB is upregulated in H9C2 cells treated with DOX. H9C2 cells were treated with different concentrations of DOX (0.25, 0.50, 1.00, 2.50 or 5.00 μmol/l), and cell viability was detected (Fig. 1A). Treatment with DOX at a dose of 1 μmol/l for 24 h decreased the cell viability to 52.11±4.14%. Thus, further experiments were performed with 1 μmol/DOX, which was consistent with the dose used in previous studies (18,19). In order to investigate whether CTSB is involved in DOX-induced cardiac injury, CTSB expression levels were assessed in an *in vitro* model following treatment with DOX. The western blotting results showed that CTSB expression levels were increased following DOX treatment compared with PBS treatment (Fig. 1B and C). Localization of CTSB was determined via immunofluorescence staining, which demonstrated that CTSB was distributed in the cytoplasm of cardiomyocytes and upregulated following DOX treatment (Fig. 1D).

CTSB deficiency attenuates DOX-induced apoptosis and oxidative stress in H9C2 cells. In order to investigate whether CTSB exerts an effect on DOX-mediated cardiac injury, CTSB siRNA was used to knock down CTSB expression levels (Fig. S1A and B). Cardiomyocyte apoptosis and oxidative stress serve key roles in DOX-induced cardiotoxicity (18). In the present study, TUNEL staining (Fig. 2A and B) showed that CTSB knockdown significantly decreased apoptosis in H9C2 cells treated with DOX, and the expression levels of the apoptosis-associated proteins Bax and c-caspase3 also decreased, whereas the expression levels of Bcl-2 increased after CTSB knockdown treated with DOX (Fig. 2D-G). In addition, ROS generation was assessed via DHE, which demonstrated that DOX treatment resulted in increased oxidative stress in H9C2 cells and that CTSB knockdown notably inhibited ROS production (Fig. 2B). In addition, western blot analysis showed that the protein expression levels of SOD1 and SOD2 were also increased in the siCTSB+DOX group compared with the siNC+DOX group (Fig. 2D, H and I). CTSB knockdown also decreased the mRNA expression levels of the NADPH oxidase subunits p67phox and GP91 and increased the expression levels of GPx in DOX-treated H9C2 cells (Fig. 2J-L).

CTSB overexpression aggravates DOX-induced apoptosis and oxidative stress in H9C2 cells. Next, it was investigated whether increased CTSB levels affected H9C2 apoptosis and oxidative stress in response to DOX. Ad-CTSB was used to overexpress CTSB in H9C2 cells (Fig. S1C and D). Fluorescence staining showed that CTSB overexpression exacerbated apoptosis and oxidative stress *in vitro* (Fig. 3A-C). The protein expression levels of Bax and c-caspase-3 increased and those of Bcl-2, SOD1 and SOD2 decreased in the Ad-CTSB+DOX group compared with the Ad-NC+DOX group (Fig. 3D-I). CTSB overexpression also increased the mRNA expression levels of the NADPH oxidase subunits p67phox and GP91 and decreased the expression levels of GPx in DOX-treated H9C2 cells (Fig. 3J-L).

CTSB mediates activation of the NF-κB pathway in response to DOX. The NF-κB pathway is associated with the apoptotic pathway, and it has dual regulatory effects in inhibiting and promoting apoptosis (20). CTSB has been found to regulate NF-κB in numerous types of cell (21,22). Thus, the NF-κB

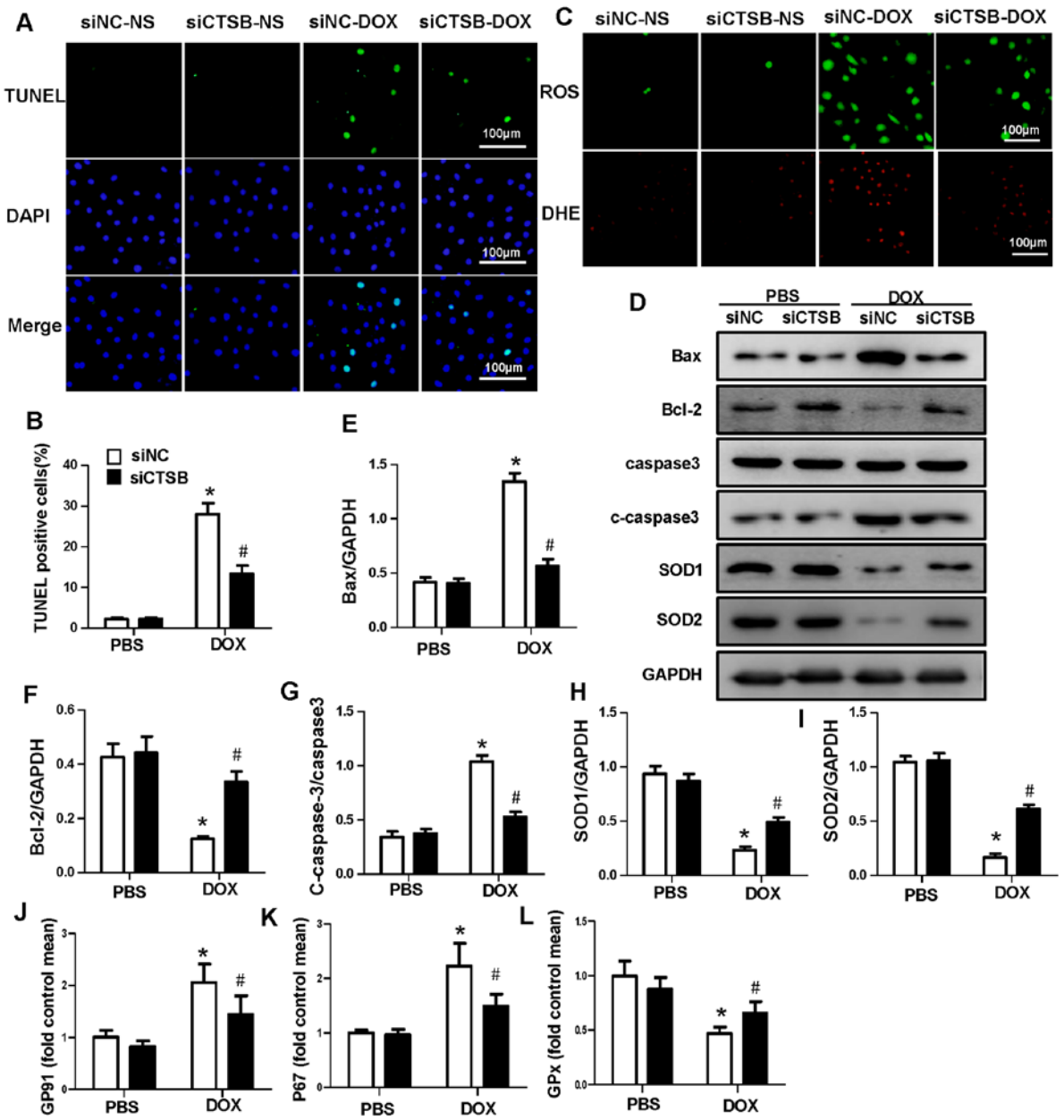

Figure 2. CTSB deficiency attenuates DOX-induced apoptosis and oxidative stress in H9C2 cells. (A) Representative images and (B) quantification of TUNEL staining. (C) Representative images of ROS and DHE staining. (D) Western blotting and quantification of expression levels of the apoptosis- and oxidative stress-associated proteins (E) Bax, (F) Bcl-2, (G) caspase-3, c-caspase-3, (H) SOD1 and (I) SOD2. mRNA expression levels of (J) GP91, (K) P67 and (L) GPx. n=6. *P<0.05 vs. siNC+PBS; #P<0.05 vs. siNC+DOX. CTSB, cathepsin B; DOX, doxorubicin; ROS, reactive oxygen species; DHE, dihydroethidium; c-caspase-3, cleaved caspase-3; SOD, superoxide dismutase; GPx, glutathione peroxidase; siNC, small interfering negative control.

pathway was investigated. The results showed that DOX treatment notably enhanced NF-κB activation. CTSB did not affect NF-κB activation at baseline, but CTSB knockdown decreased NF-κB activation in response to DOX and decreased the levels of p-NF-κB p65 and p-IκBa. Moreover, nuclear translocation of p-NF-κB p65 also decreased in response to DOX following CTSB knockdown (Fig. 4A-C and G). CTSB overexpression increased NF-κB activation and nuclear translocation following DOX treatment (Fig. 4D-F and H). Subsequently, it was determined whether CTSB lost its pro-apoptotic and pro-oxidative stress effects when NF-κB was inhibited. As expected, the NF-κB inhibitor JSH-23 mitigated DOX-induced apoptosis and oxidative stress in CTSB-overexpressing H9C2 cells, which was reflected by decreased protein expression levels of Bax and c-caspase-3 and increased protein expression levels of Bcl-2, SOD1 and SOD2 (Fig. 5A-F).

Discussion

In the present study, DOX upregulated the protein levels of CTSB in H9C2 cells, indicating that CTSB may serve a certain role in DOX-induced cardiotoxicity. Proteomic profiling of H9C2 cells in response to DOX treatment showed that CTSB was upregulated, which may be associated with NF-κB (7), but the exact mechanisms have not previously been clarified. Thus, Ad-CTSB and siCTSB were used to transfect H9C2 cells to investigate the specific role of CTSB in response to DOX, which demonstrated that CTSB overexpression exacerbated

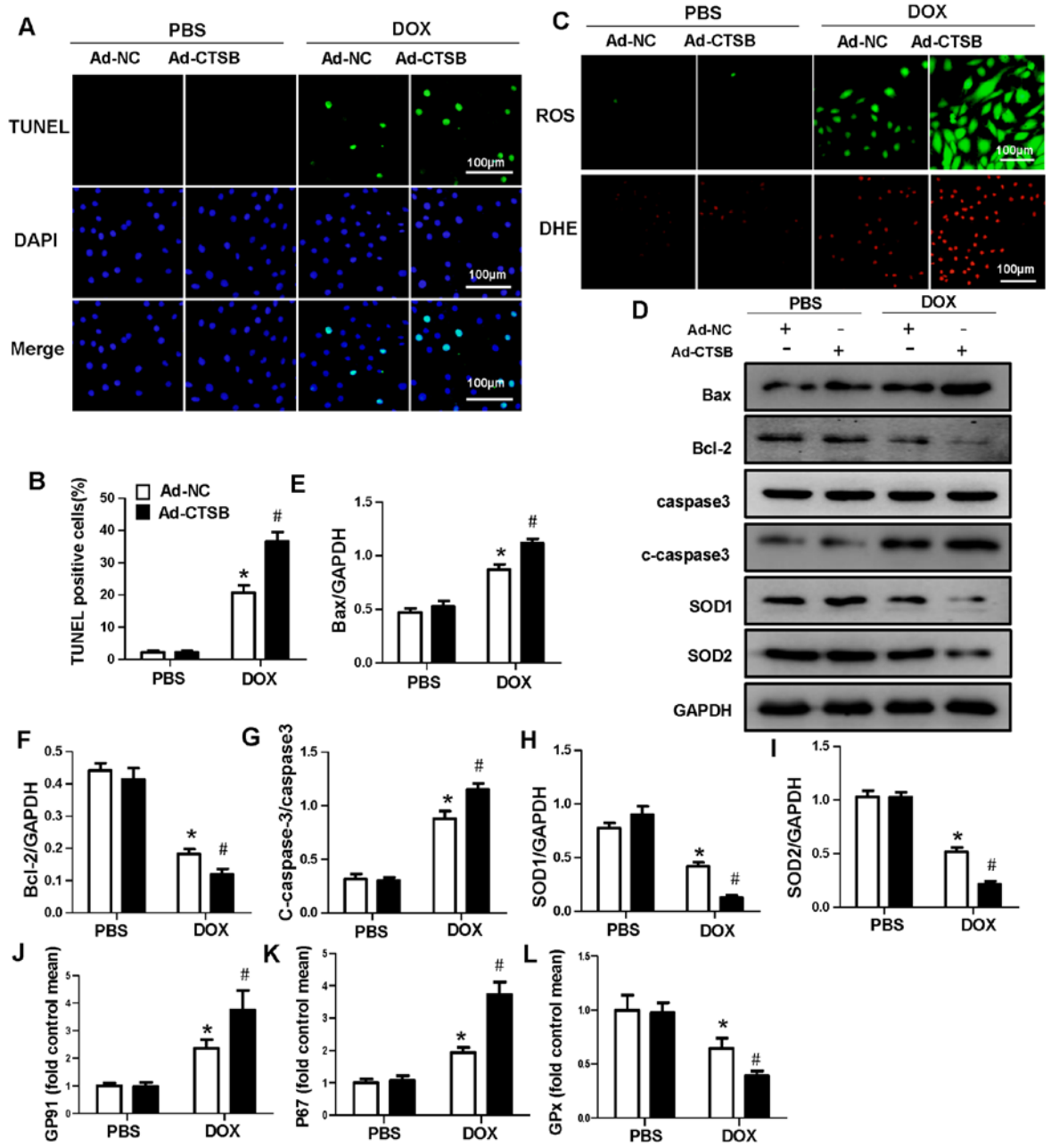

Figure 3. CTSB overexpression aggravates DOX-induced apoptosis and oxidative stress in H9C2 cells. (A) Representative images and (B) quantification of TUNEL staining. (C) Representative images of ROS and DHE staining. (D) Western blotting and quantification of expression levels of the apoptosis- and oxidative stress-associated proteins (E) Bax, (F) Bcl-2, (G) caspase-3, c-caspase-3, (H) SOD1 and (I) SOD2. mRNA expression levels of (J) GP91, (K) P67 and (L) GPx. n=6. *P<0.05 vs. Ad-NC+PBS; #P<0.05 vs. Ad-NC+DOX. CTSB, cathepsin B; DOX, doxorubicin; ROS, reactive oxygen species; DHE, dihydroethidium; c-caspase-3, cleaved caspase-3; SOD, superoxide dismutase; GPx, glutathione peroxidase; Ad-NC, adenovirus-negative control.

apoptosis and oxidative stress induced by DOX and that CTSB knockdown reversed the exacerbated phenotype of DOX-induced H9C2 injury.

Cardiomyocyte apoptosis is a notable contributor to DOX-induced cell death and can be mediated by different mechanisms, such as the AMPKα/UCP2 and FDNC5/AKT pathways (18,23,24). Growing evidence has shown that different proteolytic enzymes are involved in the regulation of apoptosis (25,26). CTSB is a protease that is localized in lysosomes under physiological conditions, and is released from lysosomes into the cytoplasm and trigger cell apoptosis via different pathways, including the activation of caspases or the release of pro-apoptotic factors from the mitochondria in response to certain stresses (27). The increase in mitochondrial membrane permeability mediates the release of cytochrome c, and it has been demonstrated that CTSB induces loss of mitochondrial membrane potential, triggers the release of cytochrome c from the mitochondria into the cytosol and activates caspase-3 in coelomocytes (28). Additionally, CTSB cleaves the pro-apoptotic Bcl-2 family member Bid, and truncated-Bid translocates to mitochondria to induce the release of cytochrome c, which triggers the activation of the apoptotic cascade (29). CTSB has been shown to be involved in apoptosis in several systems, such as hepatocytes, neurons and immune cells, and a lysosomal-mitochondrial axis theory of cell death has been proposed to indicate CTSB-regulated apoptosis (30). CTSB is

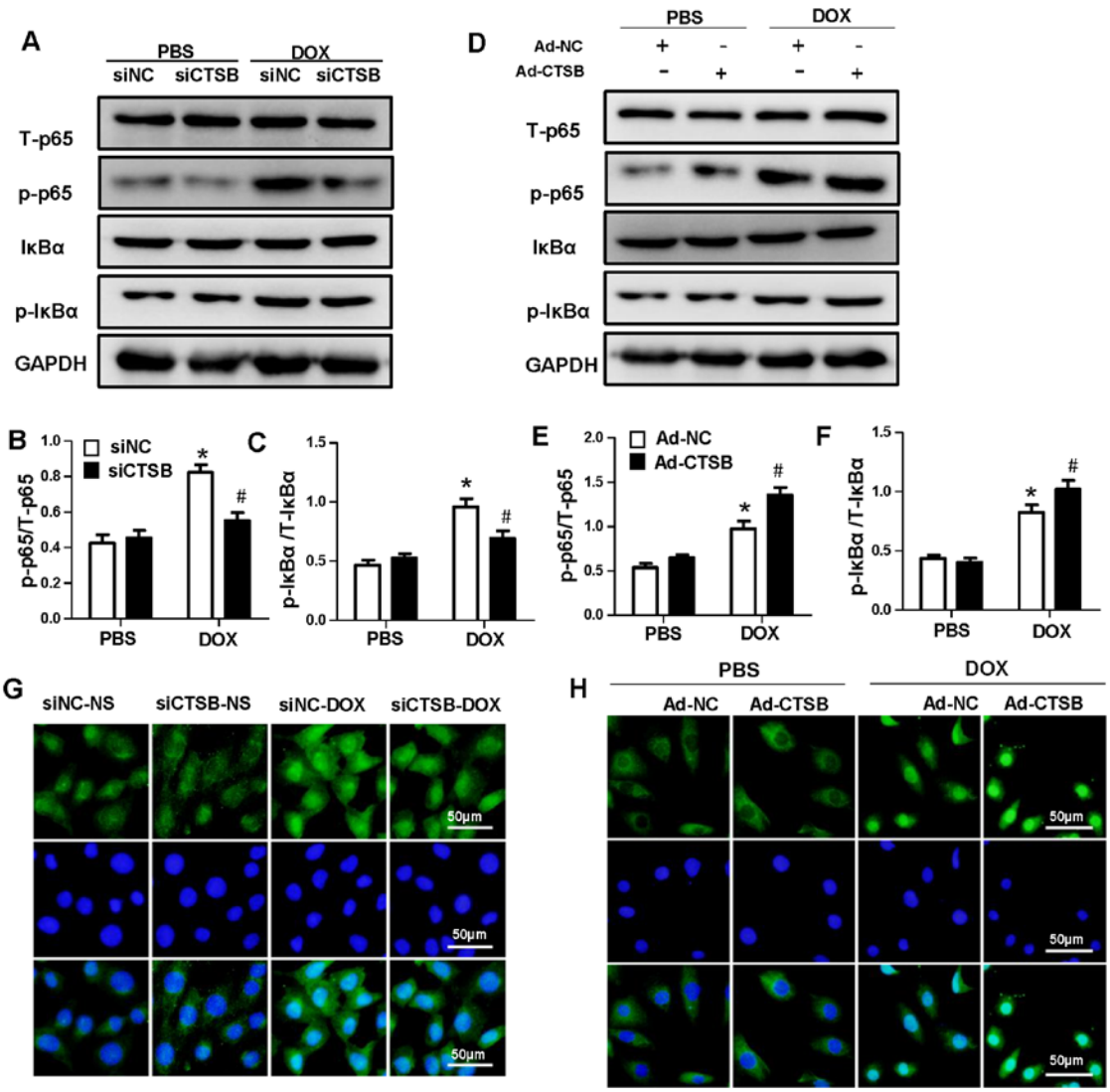

Figure 4. CTSB mediates the activation of the NF-κB pathway in response to DOX. (A) Representative western blotting and quantification analysis of (B) NF-κB p65, p-NF-κB p65, (C) IκBα and p-IκBα. *P<0.05 vs. siNC+PBS; #P<0.05 vs. siNC+DOX. (D) Representative western blotting and quantification analysis of (E) NF-κB p65, p-NF-κB p65, (F) IκBα and p-IκBα. n=6. *P<0.05 vs. Ad-NC+PBS; #P<0.05 vs. Ad-NC+DOX. Immunofluorescence staining of p-NF-κB p65 nuclear translocation in cells transfected with (G) siNC, siCTSB, (H) Ad-NC and Ad-CTSB. CTSB, cathepsin B; DOX, doxorubicin; p-, phosphorylated; si, small interfering; NC, negative control; Ad, adenovirus.

widely expressed in the myocardium (11). Wu *et al* (9) observed that CTSB participates in the regulation of stress-induced cardiomyocyte apoptosis, cardiac hypertrophy and remodelling via the TNF-α/ASK1/JNK pathway. In the present study, CTSB regulated DOX-induced H9C2 cell apoptosis, which was consistent with previous studies (7,13).

Due to its high energetic metabolic rate, the heart has the highest rate of ROS production and is susceptible to oxidative stress-associated injury. Additionally, the heart has lower levels of antioxidants and total antioxidant enzyme activity than other organs (31). Cardiac oxidative stress is associated with fibrosis, hypertrophy and decreased cardiac performance and contractility, which leads to severe cardiac dysfunction and potentially fatal cardiac events (32). CTSB mediates the regulation of oxidative stress (14). Inhibition of CTSB activity maintains the function of mitochondria and decreases the generation of ROS during *in vitro* ageing of oocytes (33). Genetic ablation of CTSB in mice significantly decreases the generation of ROS during neuroinflammation and improves cognitive impairment during ageing. In cultured microglia, inhibition of CTSB significantly decreases mitochondria-derived ROS and proinflammatory mediators induced by L-leucyl-L-leucine methyl ester (LLOMe), which is a lysosome-destabilizing agent (34). Overexpression of CTSB in microglia following treatment with LLOMe increases generation of ROS and proinflammatory mediators via impairment of mtDNA biosynthesis (34). CTSB regulates the expression levels of collagens III and IV via the toll-like receptor 2/NF-κB pathway and subsequent oxidative damage in fibroblasts (21). The present study investigated the association between CTSB and ROS in H9C2 cells and found that CTSB deficiency significantly decreased ROS generation and that CTSB overexpression increased ROS generation in DOX-induced H9C2 cytotoxicity.

The present study has demonstrated that CTSB regulates DOX-induced H9C2 cell apoptosis and oxidative stress. The underlying mechanism by which CTSB participates in DOX-induced cardiac injury was further investigated. NF-κB is a pleiotropic transcription factor that is present in almost all

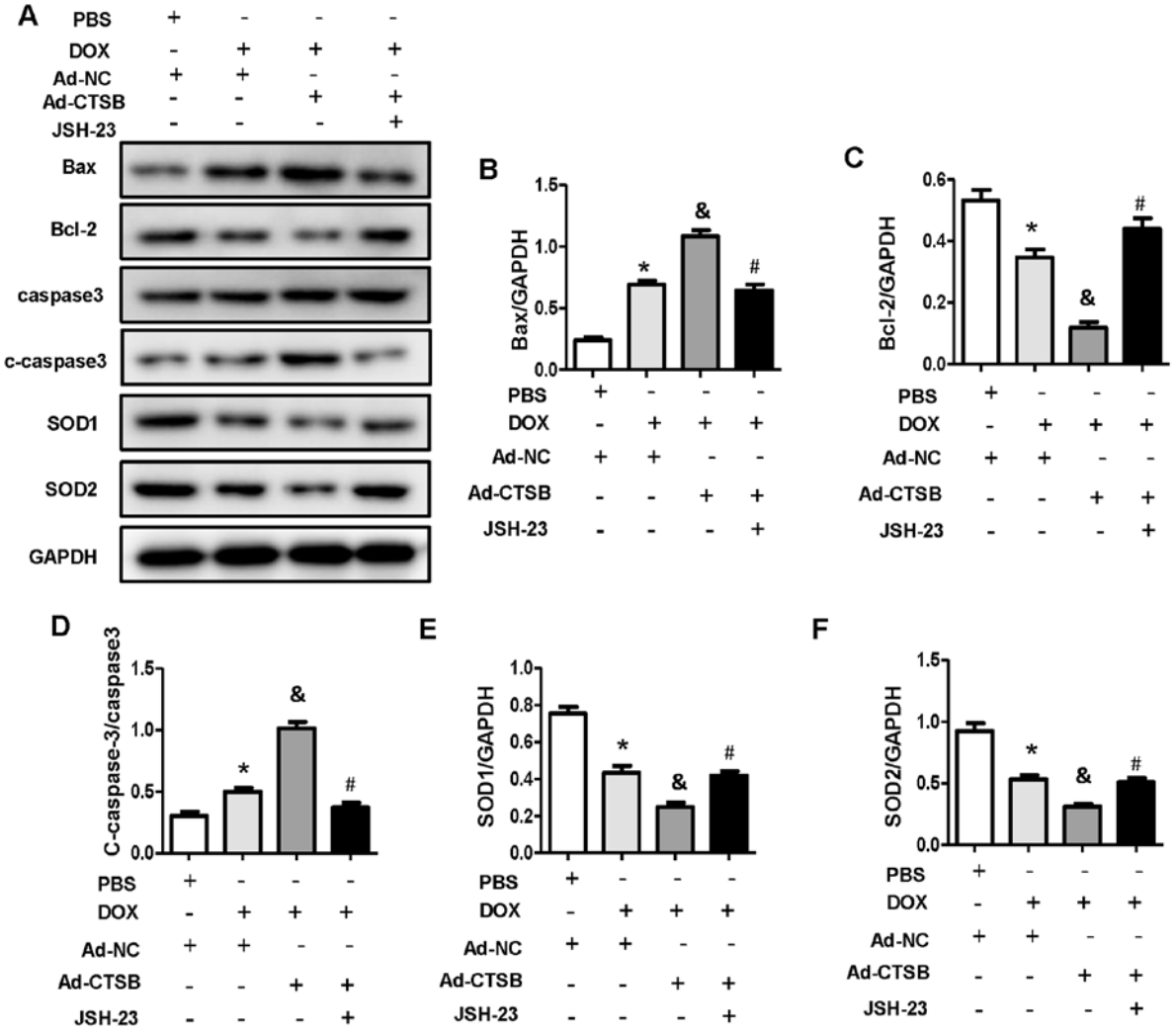

Figure 5. NF-κB inhibitor blocks the pro-apoptotic and pro-oxidative stress effects of CTSB overexpression in response to DOX. (A) Western blotting and quantification of expression levels of the apoptosis- and oxidative stress-associated proteins (B) Bax, (C) Bcl-2, (D) caspase-3, c-caspase-3, (E) SOD1 and (F) SOD2. (n=6). *P<0.05 vs. Ad-NC+PBS; &P<0.05 vs. Ad-NC+DOX; #P<0.05 vs. Ad-CTSB+DOX. CTSB, cathepsin B; DOX, doxorubicin; ROS, reactive oxygen species; DHE, dihydroethidium; c-caspase-3, cleaved caspase-3; SOD, superoxide dismutase; Ad, adenovirus; NC, negative control.

types of cell and is involved in numerous biological processes, such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (20). CTSB is responsible for NF-κB activation (35,36). CA-074Me, a specific CTSB inhibitor, prevents the activation of NF-κB via autophagic-dependent pathways in cultured microglia (35). CTSB inhibition decreases nuclear p65-NF-κB- and κB-dependent gene expression levels following lipopolysaccharide or TNF stimulation via enhancing sirtuin 1 expression levels in primary parenchymal and non-parenchymal hepatic cell types and cell lines (36). Activation of the NF-κB pathway serves a key role in the pathophysiology of multiple types of injury factor-associated cardiac dysfunction and cardiomyopathy (37,38). In the present study, DOX induced phosphorylation of IκBα and translocation of p65 NF-κB to the nucleus in H9C2 cells. Moreover, CTSB overexpression increased the phosphorylation of IκBα and the nuclear translocation of p65 NF-κB, and CTSB knockdown decreased the activation of the NF-κB pathway. The NF-κB inhibitor JSH-23 blocked the pro-apoptotic and pro-oxidative stress effects of CTSB overexpression in response to DOX. Thus, the present investigation indicated that CTSB may mediate NF-κB activation to regulate H9C2 cell apoptosis and oxidative stress. However, NF-κB is also sensitive to ROS; it has been proven that ROS activate IKK, thus promoting the activation of NF-κB (39). ROS and NF-κB activation may mutually regulate the mechanism of DOX-induced cardiotoxicity.

In conclusion, CTSB may be a potential therapeutic agent for the treatment of DOX-induced cardiotoxicity.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CL and ZC conceptualised the study design. LZ designed the experiments. CL analysed the data. CL, ZC, TH and QY performed the experiments. CL drafted the manuscript. LZ and ZC reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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