Thrombopoietin protects H9C2 cells from excessive autophagy and apoptosis in doxorubicin-induced cardiotoxicity

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Abstract. Cardiac toxicity has been the major concern when using doxorubicin (DOX) in cancer therapy. Thrombopoietin (TPO) protects cardiac cells from DOX-induced cell damage; however, its molecular mechanism remains exclusive. The anti-autophagic and anti-apoptotic effects of TPO upon DOX treatment were studied in the cardiac H9C2 cell line, with bafilomycin A1 treatment as a positive control for autophagy inhibition. Cell viability was measured by Cell Counting Kit-8 assay in different treatment groups. The mRNA and/or protein levels of apoptotic markers and autophagy-associated factors were detected. The mean number of microtubule-associated protein 1A/1B-light chain 3 (LC3) puncta per cell was quantified to indicate autophagosomes and autolysosomes, of which the ones co-stained with lysosomal-associated membrane protein 1 were considered as autolysosomes. DOX treatment (5 μg/ml, 24 h) significantly impaired H9C2 cell viability compared with the control, while TPO pretreatment (10 ng/ml, 36 h) improved cell viability upon DOX treatment. DOX exposure markedly increased LC3 puncta in H9C2 cells, and TPO pretreatment reduced the number of autophagosomes, but showed no significant inhibitory effect on autolysosome formation. The autophagy inhibition by TPO upon DOX treatment was confirmed according to protein quantification of LC3-II and nucleoporin 62. TPO also suppressed autophagy-promoting protein Beclin-1, and elevated the anti-autophagic factors GATA-binding protein-4 and B cell lymphoma-2. Furthermore, TPO reduced DOX-induced apoptosis in H9C2 cells, as reflected by the amount changes of caspase-3. Taken together, these results revealed that TPO has a protective role in H9C2 cells from DOX-induced autophagy as well as apoptosis, and indicated that TPO may act as a cardioprotective drug in DOX-treated patients.

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Introduction

Doxorubicin (DOX) is one of the most widely used and successful antitumor drugs; however, it has a number of serious side effects, including hematopoietic suppression, nausea, vomiting, extravasation and alopecia, of which cardiac toxicity has been the major concern with its use in cancer treatment (1). Cardiac toxicity may be present following DOX chemotherapy as symptoms ranging from asymptomatic electrocardiography-changes to pericarditis and decompensated cardiomyopathy (2). The mechanisms by which DOX causes cardiac toxicity have been widely investigated (2).

Cardiac toxicity from DOX involves changes in the high-energy phosphate pool, endothelin-1 levels and disturbances of myocardial adrenergic signaling (2). An increase in cardiac oxidative stress is also associated with DOX-induced cardiomyopathy through damage, including lipid peroxidation, reduced antioxidant levels and fewer sulfhydryl groups (3). Previous studies have established that DOX-induced myocardial apoptosis performs a role in the development of heart failure (4,5). The DOX-induced cardiac toxicity causes myofibrillar deterioration through cardiomyocyte apoptosis (6), endothelial cell apoptosis (7) and intracellular calcium dysregulation (8). The level of apoptosis can be measured by monitoring caspase-3, one of the major executors of apoptosis (9).

It has been suggested that dysregulation of autophagy performs an important role in DOX-induced cardiotoxicity; however, the detailed mechanisms were less well understood (10). DOX-induced cardiomyocyte autophagy may be protective or detrimental depending on the dosage of DOX (11). However, it is now speculated that cardiomyocyte autophagy may be one of the major mechanisms of cardiotoxicity in itself (12,13).

Autophagy is an essential process for optimal cellular function and survival, where damaged or unwanted proteins and organelles are removed from the cell (14). Autophagy may be stimulated in order to protect the cell from stress stimuli or, alternatively, to contribute to cell death (10). Cardiac autophagy is a maladaptive response to hemodynamic stress (15). An

increased number of autophagosomes have been observed in cardiac tissues from patients with aortic stenosis (16) and dilated cardiomyopathy (17). Autophagy serves a role in cardiomyocyte salvage (18) and it is essential for cardioprotection when induced by ischemic preconditioning (19,20). It also performs distinct roles during ischemia and reperfusion. It may be protective during ischemia, whereas detrimental during reperfusion (21). The Beclin 1-Vps34 complex was pivotal in driving autophagy, which is required for autophagosome formation; autophagy related 14 (ATG14L) enhances vacuolar protein sorting 34 (Vps34) activity in a Beclin 1-dependent manner, thus positively regulating autophagy, while Rubicon suppresses autophagosome maturation through inhibiting Vps34 activity (22-24). There are a number of ways of measuring autophagy. As microtubule-associated protein 1A/1B-light chain 3 (LC3)-II is consistently kept in the membrane of the autophagosome until it fuses with the lysosomal membrane, LC3-II is used as a marker of autophagosomes (25). The formation of the autophagosome membrane is followed by its subsequent fusion with the lysosome via lysosomal-associated membrane proteins (LAMP1 and LAMP2), as well as Rab7 and UV radiation resistance-associated gene (3). Co-localization of LC3-II and LAMP reflects the fusion of autophagosome and lysosomal, that is to say, autophagosome maturation (26). Finally, nucleoporin 62 (p62) can be used to monitor autophagic flux, since total cellular expression levels of p62 are inversely associated with autophagic activity (27).

Previous studies have shown that there is cross talk between autophagic and apoptotic pathways, including inducer and signaling pathways (28,29). Autophagy protein 5 (Atg5), which was previously characterized as a protein specifically required for autophagy, can directly lead to cell death by activating the apoptotic pathway without the activation of autophagic pathways (30,31). Furthermore, B cell lymphoma-2 (Bcl-2) and B cell lymphoma-extra-large, the well-characterized anti-apoptotic proteins, appear to be important modulators of autophagy through binding and inhibiting Beclin 1 (32). Preservation of zinc finger transcription factor GATA-binding protein-4 (GATA-4) has been shown to protect cardiomyocytes from DOX-induced toxicity by inhibiting apoptosis and autophagy through regulating expression of Bcl-2 positively, and Beclin-1 negatively (5,33). However, the cross talk between autophagy and apoptosis is complex. In certain conditions, autophagy can suppress apoptosis, and in others, autophagy can induce apoptosis (34). The interaction of autophagy and apoptosis in the background of DOX-induced cardiac toxicity requires further exploration.

Our previous study provided the first evidence that thrombopoietin (TPO) is a protective factor against DOX-induced cardiotoxicity, since it was demonstrated that TPO reduced DOX-induced apoptosis of H9C2 cells (35). TPO is a cytokine produced by liver and kidney and regulates the production of platelets. It promotes megakaryocytic/platelet lineage, angiogenesis and inhibits apoptosis (36,37). Other studies are in agreement with the present results (38,39). A study reported that erythropoietin (EPO), which is homogenous with TPO, regulates autophagy via the protein kinase B (Akt)/mechanistic target of rapamycin signaling pathway (40). However, the regulation of TPO on autophagy has not been reported. TPO shares homology with EPO (41) and their function is also similar. Since EPO is also a heart protective factor (42,43), it was proposed that TPO may also regulate autophagy.

Therefore, in the present study, the hypothesis that TPO may perform protective roles in cardiac cells against DOX-induced autophagy and apoptosis was investigated. The research model was established in H9C2 cells, the cardiomyocyte cell line that is often used in studies on cardiomyocyte function (44), and was used in our previous study (35). Bafilomycin A1 (BFA) treatment was used as a positive control for autophagy inhibition, since BFA is a specific inhibitor of vacuolar type H^+-ATPase in cells that may effectively hinder fusion between autophagosomes and lysosomes (45).

Materials and methods

Cell culture. Rat myoblast H9C2 cell line (American Type Culture Collection; cat. no. CRL-1446) was purchased from the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific. Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.), 2 mM glutamine, 100 IU penicillin and 100 μg/ml streptomycin (46). Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

Drug treatments. TPO (3SBio Inc., Shenyang, China) was dissolved in saline to make a stock solution of 25 μg/ml and then diluted to make a final concentration of 10 ng/ml for subsequent experiments. DOX (Sangon Biotech Co., Ltd., Shanghai, China) was dissolved in saline to make a stock solution of 25 mg/ml and then diluted to make a final concentration of 5 μg/ml for subsequent experiments. This dose was used with reference to our previous study, where different doses of DOX and their effects on H2C9 cells were investigated (35). BFA (Sangon Biotech Co., Ltd.) was dissolved in saline to make a stock solution of 100 μg/ml and then diluted to make a final concentration of 10 nM for autophagy inhibition. Cells were pretreated with TPO for 36 h, and then treated with DOX for 24 h, or treated with DOX for 24 h, and then with BFA for 6 h.

Western blot analysis. Cells were lysed in 200 μl of Laemmli buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The protein concentration was determined by the Bradford method. The protein was then boiled in 1X loading buffer for 5 min and electrophoresed on 12% SDS-PAGE. Total protein (30 μg) was loaded per lane and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 2 h at 37˚C with 5% skimmed milk powder. The membranes were incubated with primary antibodies overnight at 4˚C. Following three washes with TBST, the immobilized protein samples were then incubated with horseradish peroxidase secondary antibodies (cat. no. A0208; Beyotime Institute of Biotechnology, Shanghai, China; A0208, dilution, 1:1,000) for 1 h at 4˚C, and the protein complex was detected using the enhanced chemiluminescence substrate system (Pierce Biotechnology, Inc., Rockford, IL, USA). In particular, the blotting and imaging of Bcl-2 and GATA-4 were performed with a full-automatic western blot system (Protein Simple, San Jose, CA, USA).

The following primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): p62 (cat. no. 5114, dilution, 1:1,000), Beclin-1 (cat. no. 3495, dilution, 1:1,000) and caspase-3 (cat. no. 9665, dilution, 1:1,000). The following primary antibodies were purchased from Abcam (Cambridge, UK): LAMP1 (cat. no. ab24170, dilution, 1:1,000), Bcl-2 (cat. no. ab59348, dilution, 1:500) and GATA-4 (cat. no. ab134057, dilution, 1:500). LC3 was purchased from Thermo Fisher Scientific, Inc. (cat. no. PA1-16930, dilution, 1:500). β-actin (cat. no. 20536-1-AP, dilution, 1:1,000) and GAPDH (cat. no. 10494-1-AP, dilution, 1:1,000) antibodies were purchased from Proteintech Group (Rosemont, IL, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from H9C2 cells, using RNA extraction buffer E-Z 96 total RNA kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's protocol. The RT kit was RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Temperature protocol was 42˚C.

The sequences of all forward and reverse primers, including the sequence of the reference gene primer are presented below: R-GAPDH forward, 5'-GCTCTCTGCTCCTCCCTGTTCT-3' and reverse, 5'-GCCAAATCCGTTCACACCGACCT-3'; R-LC3 forward, 5'-GCACTACGGCTGCTCTTTATAC-3' and reverse, 5'-CACAGATCTTCAACAGCACAGT-3'; R-Beclin 1 forward, 5'-CAGGATGGTGTCTCTCGAAGA-3' and reverse, 5'-CCCCGATCAGAGTGAAGCTAT-3'; R-ATG14L forward, 5'-CCGAACAATGGGGACTACTCT-3' and reverse, 5'-TGG TGTAGGCAGGGTTGTTAT-3'; R-Rubicon forward, 5'-TCC CAGTTCAGTTCACGTGA-3' and reverse, 5'-TGTAGGAAG CAGTGCGAGAA-3'; R-Caspase-3 forward, 5'-TGGACT GCGGTATTGAGACA-3' and reverse, 5'-GCGCAAAGT GACTGGATGAA-3'; R-Bcl-2 forward, 5'-AACTCTTCA GGGATGGGGTG-3' and reverse, 5'-CACAGAGCGATG TTGTCCAC-3'; and R-GATA-4 forward, 5'-CTAACCTT ACTGGCCGTAGC-3' and reverse, 5'-GGGAGAACAGC GTAAATGA-3'.

RT-qPCR analysis for autophagy-associated genes was performed using an ABI StepOnePlus™ sequence detection system (Thermo Fisher Scientific, Inc.). The LightCycler amplification of PCR products was detected with SYBR-Green I dye (Roche Diagnostics, Basel, Switzerland). PCR cycling conditions were 95˚C for 10 min and 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, and 72˚C for 15 sec, followed by a final melting curve program (95˚C for 15 sec, 60˚C for 60 sec, and 95˚C for 15 sec). Relative expression levels of target genes of interest were calculated using the $2^{-\Delta\Delta Cq}$ method, and GAPDH was used as an internal control (47). Each sample was investigated in triplicate and mean values were used for quantitation.

Cell proliferation and cytotoxicity assay. Cell viability was measured using Cell Counting Kit-8 (CCK-8; 5 mg/ml; Beyotime Institute of Biotechnology) for 3 h according to the manufacturer's protocol. The absorbance was then measured at a wavelength of 492 nm using a microplate reader.

Autophagosome formation. The LC3-green fluorescent protein (GFP) lentivirus expression vector was produced as previously described (48). H9C2 cells were infected with LC3-GFP lentivirus in the presence of polybrene (6 μg/ml). Lentivirus-infected cells were selected with blasticidin (5 μg/ml) and maintained for 10 days in medium containing blasticidin. Stably-infected, blasticidin-resistant H9C2 cells were cloned and expanded as previously described (26). Following different treatments, H9C2 cells were washed three times in PBS and fixed with 4% paraformaldehyde in PBS (Guangzhou Chemical Reagent Factory, Guangzhou, China) for 10 min at room temperature. H9C2 cells were observed at x650 magnification laser scanning confocal microscopy (IX81 + FV10-M CPSU + IX2-UCB + U-RFL-T; Olympus Corporation, Tokyo, Japan). GFP-LC3 puncta represented the autophagosome. Autophagy was analyzed by quantifying the mean number of GFP-LC3 puncta per cell in all cells in the population by using Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA).

Immunostaining. Cells transfected with LC3-GFP vectors were seeded on coverslips at a density of $1x10^4$ cells/ml), and fixed with PBS solution containing 4% paraformaldehyde for 10 min at room temperature (25˚C). The coverslips were blocked with 10% normal goat serum (Vector Labs, Burlingame, CA, USA) at room temperature (25˚C) for 60 min. The coverslips were then incubated with LAMP1 antibody (cat. no. ab24170, dilution, 1:200; Abcam) at 4˚C overnight, and the following day with Alexa Fluor-conjugated secondary antibody (ab150077, dilution 1:1,000; Abcam, Shanghai, China) at 37˚C for 30 min. Finally, fluorescent mounting media containing DAPI was added. The results were observed at x100 magnification laser scanning confocal microscopy (IX81 + FV10-MCPSU + IX2-UCB + U-RFL-T; Olympus Corporation).

Hematoxylin and eosin (H&E) staining. Following drug treatment, H9C2 cells were incubated in 4% paraformaldehyde for 20 min at room temperature (25˚C), and washed with PBS followed by hematoxylin solution incubation for 10 min at room temperature (25˚C). Cells were next washed in running tap water for 10 min, immersed in distilled water briefly and in 95% alcohol for 5 sec. The cells were then counterstained in eosin solution for 30 sec to 2 min at room temperature (25˚C). Cell morphology was examined under a x40 light microscope.

Statistical analysis. Data were analyzed by SPSS 19.0 statistical software (IBM SPSS, Armonk, NY, USA). Data are presented as the mean ± standard deviation. Statistical significance was estimated by one-way analysis of variance with least significant difference post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TPO increases viability of DOX-treated cardiomyocytes. The CCK-8 assays demonstrated that DOX treatment (5 μg/ml, 24 h) significantly impaired H9C2 cell viability (reduced by 27.1±1.1%, n=3; $P<0.001$; Fig. 1A) when compared with the control, while TPO pretreatment prior to DOX significantly improved cell viability (increased by 7.8±0.3%, n=3; $P<0.001$; Fig. 1A) when compared with the DOX treatment group. These results indicated that TPO can protect cardiomyocytes from DOX-induced growth inhibition.

TPO reduces DOX-induced autophagy in cardiomyocytes. The mean number of GFP-LC3 puncta per cell was counted in all cells in each field (Fig. 1B). There were negligible GFP-LC3 puncta in the control cells, while DOX markedly increased the GFP-LC3 puncta, indicating that DOX was able to induce cardiomyocyte autophagy (n=3; control 5±5.0 vs. DOX 130±14.0; P<0.001). H9C2 cells were treated with the lysosomal inhibitor BFA to block autolysosome formation, and the autophagosome formation was significantly increased compared with the DOX alone treated cells (n=3; DOX 130±14.0 vs. DOX+BFA 340±32.2; P<0.001). However, the mean number of GFP-LC3 puncta per cell was significantly decreased by TPO pretreatment compared with DOX alone, indicating the ability of TPO to inhibit DOX-induced autophagy (n=3; DOX 130±14.0 vs. DOX + TPO 98±9.6; P<0.05).

TPO regulates autophagy-associated proteins. The mRNA and protein levels of LC3-II and Beclin 1 were all upregulated by DOX treatment (n=3; all P<0.001; Fig. 2), and were further increased by lysosomal inhibitor BFA (P<0.01 or P<0.001). However, TPO pretreatment prior to DOX treatment reduced the mRNA level of LC3 and Beclin 1 compared with the DOX-treated alone group, which was consistent with the protein expression (P<0.01 or P<0.001). DOX treatment significantly decreased ATG14L and Rubicon mRNA levels compared with the control (n=3; both P<0.001; Fig. 2). BFA post-treatment and TPO pretreatment lead to a significant increase in ATG14L mRNA upon DOX treatment (P<0.05 or P<0.01; Fig. 2A).

The protein amount of p62 was significantly decreased in DOX-treated cells compared with the control cells (P<0.05), and increased following BFA addition compared with the DOX single treatment; TPO-pretreated H9C2 cells compared with non-TPO-pretreated H9C2 cells also showed increased p62 levels following exposure to DOX (P<0.001; Fig. 2B).

The expression of anti-autophagic proteins was also detected. GATA-4 mRNA was decreased 18-fold following DOX treatment (n=3; P<0.001; Fig. 2A); neither TPO nor BFA could upregulate GATA4 expression upon DOX treatment. However, at protein level, both the BFA post-treatment and TPO pretreatment showed significantly increased GATA-4 compared to DOX alone treated cells (n=3; P<0.01 or P<0.001; Fig. 2B). Furthermore, although BFA and TPO had no effect on Bcl-2 mRNA level upon DOX treatment, it significantly increased the protein level (n=3; P<0.05 or P<0.001; Fig. 2B).

Taken together, these results confirmed that TPO reduced DOX-induced autophagy in H9C2 cells.

TPO has no significant effect on autophagosome maturation. LAMP1 protein level significantly increased following DOX treatment compared with the control, indicating that DOX may regulate lysosome function (n=3; P<0.001; Fig. 3A). However, TPO had no effect on LAMP expression compared with DOX treatment alone. In order to assess the effect of TPO on autophagosome maturation, the co-localization of GFP-LC3-labelled autophagosomes with LAMP1-stained lysosomes was measured. The number of LC3^{+}/LAMP1^{+} co-stained autophagosomes was higher in each of the DOX-treated groups compared with that in

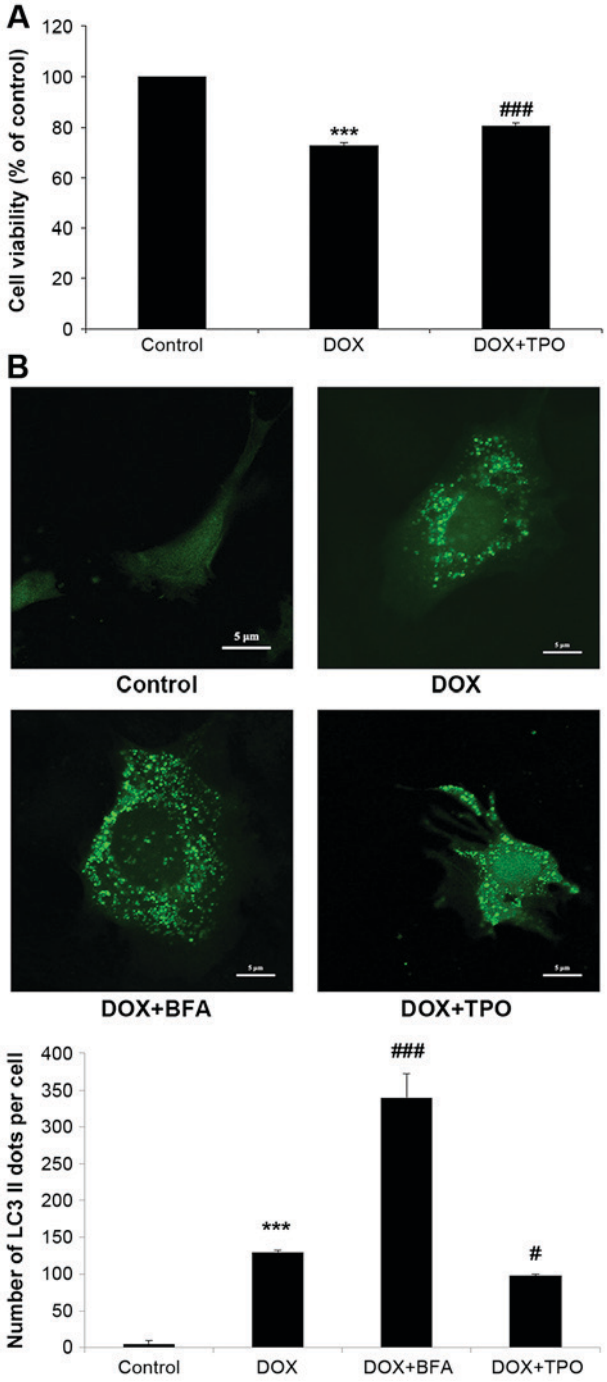

Figure 1. TPO increased the viability of H9C2 cells after DOX treatment and reduced DOX-induced autophagy. H9C2 cells were divided into four groups. Control group was untreated. DOX groups were all incubated in the presence of DOX (5 μg/ml, 24 h); besides, DOX + BFA group was subsequently treated with BFA (10 nM, 6 h), while DOX + TPO group pretreated with TPO (10 ng/ml, 36 h). (A) The viability was estimated by Cell Counting Kit-8 assay. (B) H9C2 cells were transduced with LC3-GFP lentivirus. Representative fluorescent images were shown. Scale bar, 5 μm. Autophagosomes were calculated as the mean number of GFP-LC3 puncta per cell for all cells in the population. Results are presented as the mean ± standard deviation and were estimated by one-way analysis of variance with least significant difference post-hoc test (n=3). ***P<0.001 vs. control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. DOX treated alone group. TPO, thrombopoietin; DOX, doxorubicin; BFA, bafilomycin A1; GFP, green fluorescent protein; LC3, microtubule-associated protein 1A/1B-light chain 3.

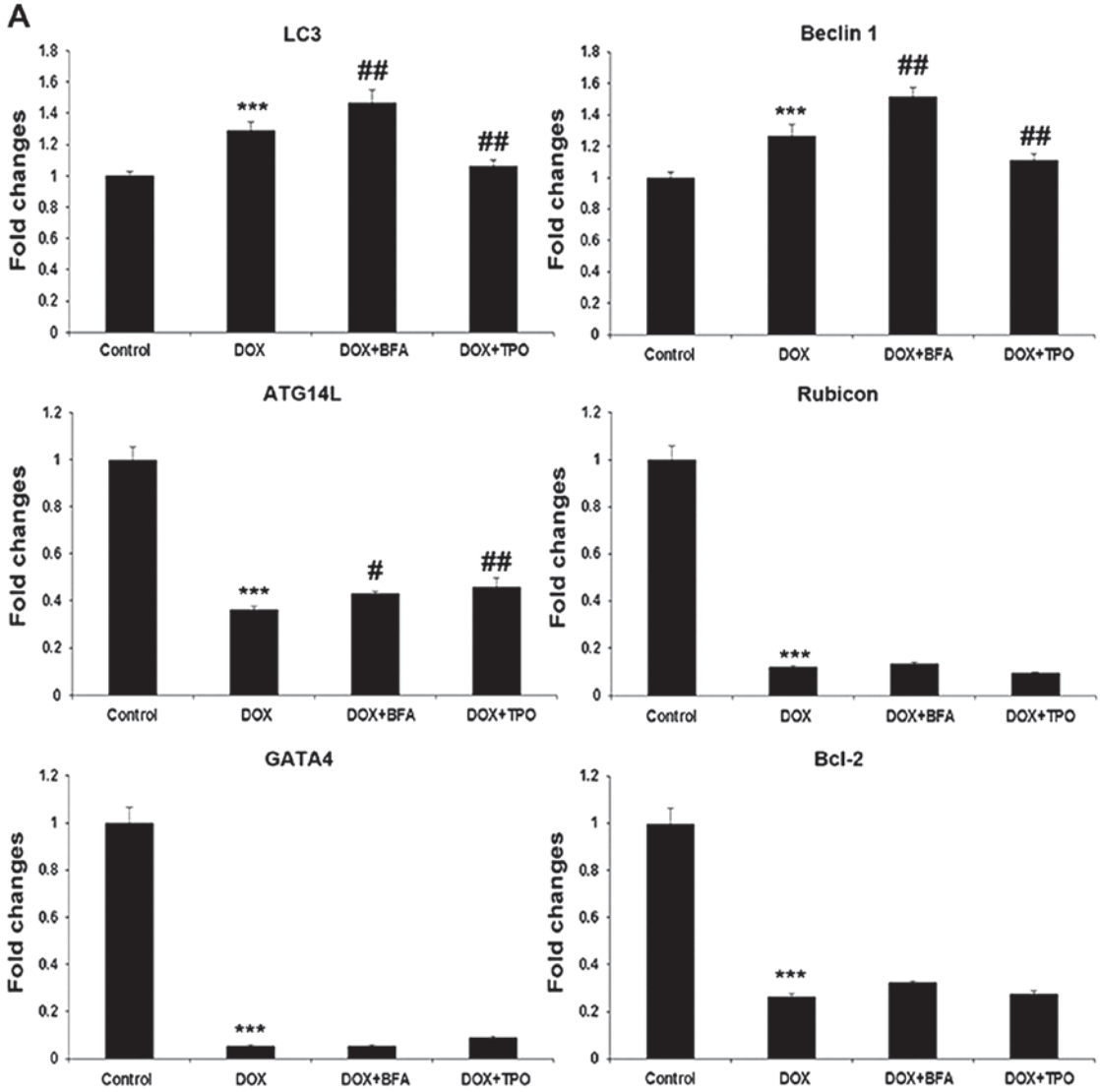

Figure 2. TPO pretreatment reduced autophagy-promoting factors while increased autophagy-inhibitory factors. Grouping was set the same as described in Fig. 1. (A) Reverse transcription-quantitative polymerase chain reaction for the genes encoding LC3, Beclin 1, ATG14L, Rubicon, GATA-4 and Bcl-2.

the control group. BFA addition reduced LC3 and LAMP1 co-localization, indicating that BFA blocked DOX-induced autophagosome maturation. However, the numbers of $LC3^{+}/LAMP1^{+}$ co-stained autophagosomes in the DOX + TPO groups were not statistically significant different from the DOX group, indicating that TPO did not block DOX-induced autophagosome maturation (Fig. 3B).

TPO treatment reduces apoptosis in H9C2 cells. In the H&E stained specimens of H9C2 cells, the morphology of control cells was not altered, whereas cells in the DOX-treated group decreased in size and altered to a round shape. With TPO or BFA treatment, a number of cells were restored to their normal morphology (Fig. 4A). The mRNA and protein levels of caspase-3 in DOX + TPO-treated groups were significantly reduced compared with that in the DOX-treated alone group (Fig. 4B and C; both $P<0.001$). The DOX + BFA-treated groups showed the same change (Fig. 4B and C; both $P<0.001$). Taken together, these results demonstrated that in DOX-treated H9C2 cells, the inhibition of autophagy reduced apoptosis.

Discussion

In the present study, it was revealed that TPO could protect H9C2 cells from DOX-induced excessive autophagy and apoptosis. These results explored a novel mechanism of the cardioprotective function of TPO on doxorubicin-induced myocardial damage. To the best of our knowledge, this is the first study to demonstrate that apart from apoptosis, TPO may significantly reduce DOX-induced autophagy.

DOX-induced cardiotoxicity has been a major limitation on its usage in cancer therapy (1). TPO has been approved to exert cardioprotective effects when used with DOX in cancer therapy (35). Although mechanisms of its cardioprotective effects remain unclear, previous studies have indicated that TPO regulates the expression of numerous genes, including genes involved in apoptosis, Akt and extracellular signal-regulated kinase pathways, cell division regulators, blood vessel remodeling and matrix, channel regulators, muscle and contractile proteins (37). The present study firstly demonstrated that TPO also regulates DOX-induced autophagy in H9C2 cells. The present data confirmed that autophagy and

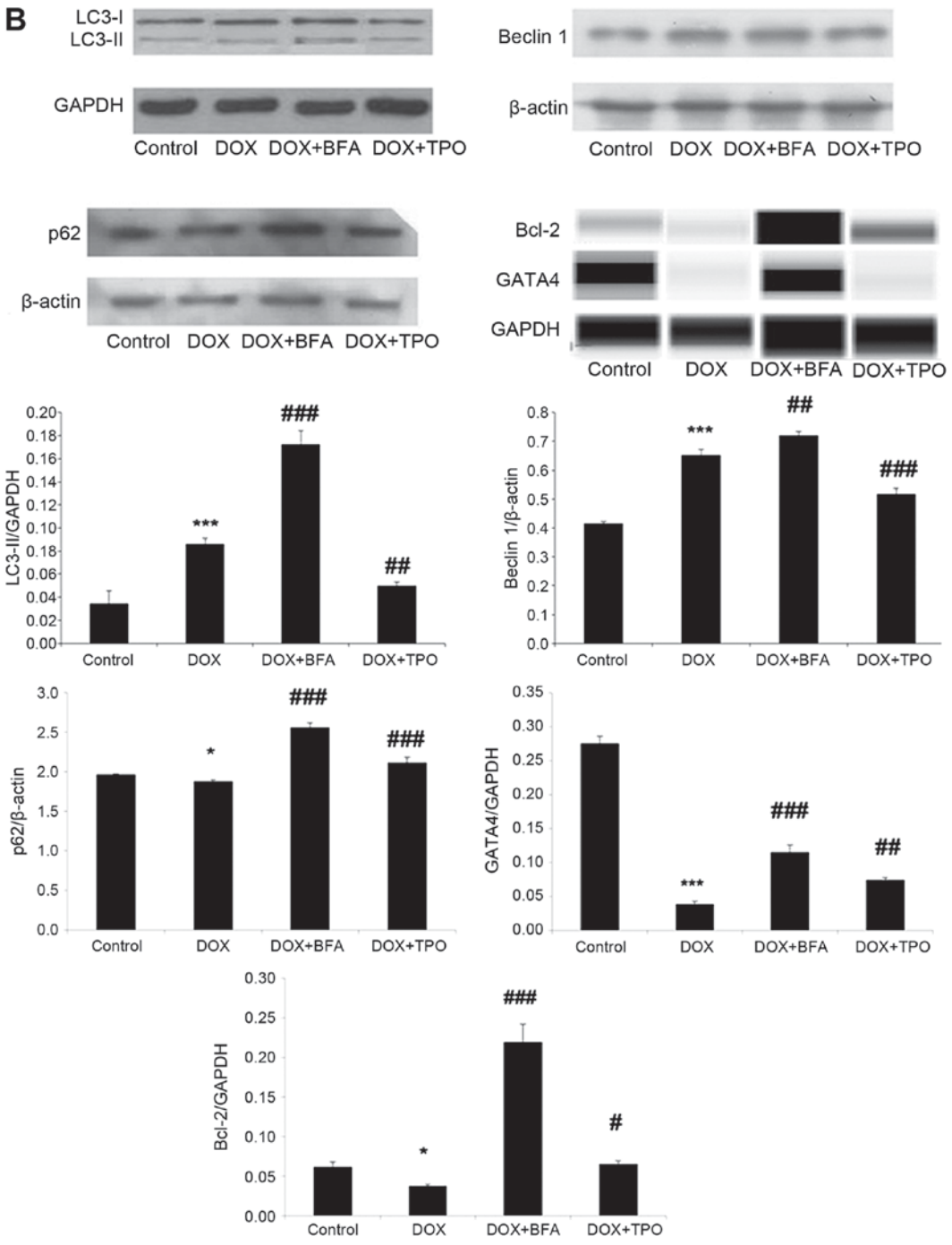

Figure 2. Continued. (B) Western blots: Representative images and quantification histograms for LC3-I/II, Beclin 1, p62, GATA-4 and Bcl-2. Results are presented as the mean ± standard deviation and were estimated by one-way analysis of variance with least significant difference post-hoc test (n=3). *P<0.05 and ***P<0.001, compared with control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. DOX treated alone group. LC3, microtubule-associated protein 1A/1B-light chain 3; GATA-4, GATA-binding protein-4; Bcl-2, B cell lymphoma 2; p62, nucleoporin 62; DOX, doxorubicin; ATG14L, autophagy related 14; TPO, thrombopoietin; BFA, bafilomycin A1.

apoptosis are upregulated in H9C2 cells following DOX treatment, whereas TPO inhibits DOX-induced autophagy and apoptosis.

Autophagy is a conserved cellular process involving the degradation of a cell's own components, which may be either protective or detrimental in DOX-treated cells depending on the stress level (21). A number of studies have revealed that DOX treatment can increase autophagy in cardiac cells, which mediates DOX-induced cardiotoxicity (12,13). In the present study, DOX increased H9C2 autophagy was indicated by the accumulation of GFP-LC3 puncta and increased expression of Beclin 1, which is in agreement with other studies (5,49). Notably, it was demonstrated that upon DOX treatment, TPO inhibits H9C2 cell autophagy as shown by the decreased expression of GFP-LC3 puncta, LC3II, Beclin 1 and increased amount of p62 compared with the DOX alone-treated groups.

In the present study, the regulatory effect of BFA upon DOX-induced autophagy was not completely consistent with that of TPO. BFA and TPO promoted the accumulation of p62

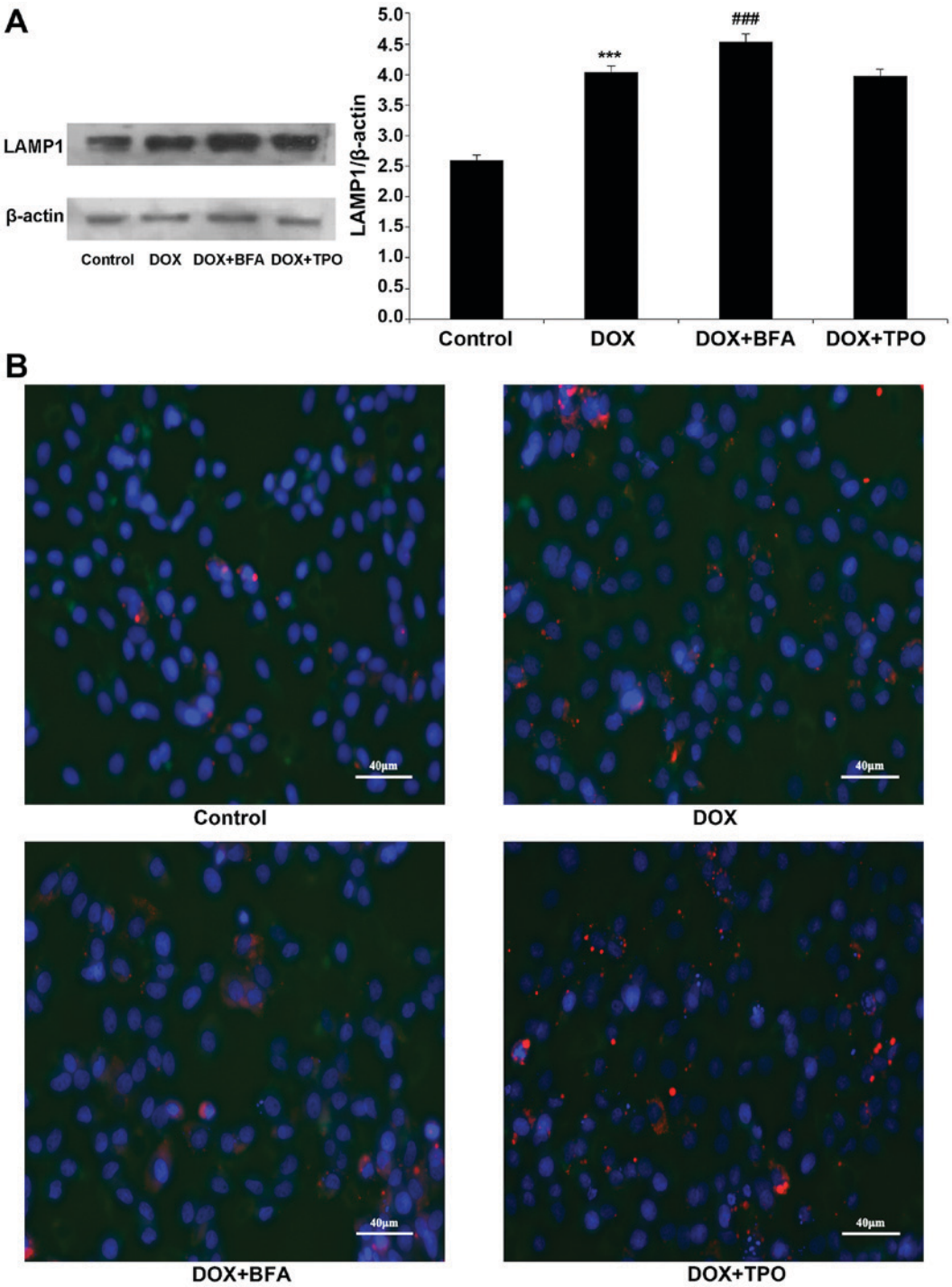

Figure 3. TPO pretreatment did not show a significant inhibitory effect on autophagosome maturation. Grouping was set the same as described in Fig. 1. (A) Western blots: Representative images and quantification histograms for LAMP1. (B) Monitoring autophagosome maturation in H9C2 cells: The cell nuclei are stained blue with DAPI. The GFP-LC3 puncta represented the autophagosome. The LAMP1 stained lysosomes were red. Scale bar, 40 μm. Results are presented as the mean ± standard deviation and were estimated by one-way analysis of variance with least significant difference post hoc test (n=3). ***P<0.001; $^{###}$P<0.001 vs. DOX treated alone group. DOX, doxorubicin; LC3, microtubule-associated protein 1A/1B-light chain 3; TPO, thrombopoietin; LAMP1, lysosomal-associated membrane protein 1; GFP, green fluorescent protein; BFA, bafilomycin A1.

upon DOX treatment, indicating that the ultimate outcome of their regulation on autophagy was inhibitory. However, BFA significantly increased GFP-LC3 puncta compared with DOX treatment alone, which was in accordance with the well-accepted conclusion that BFA suppresses autophagic degradation through inhibiting the fusion between autophagosomes and lysosomes, which then leads to an increase in autophagosomes (12,13); while TPO has no such effect on autophagosomes. It was confirmed that BFA blocked the DOX-induced autophagosome maturation by detecting co-localization of LC3 and LAMP (26). LAMP1 is an important lysosomal membrane protein, which is expressed at high levels in a number of normal tissue cells and is responsible in part for maintaining lysosomal integrity (50). It was revealed that TPO did not affect the appearance of $LC3^{+}/LAMP1^{+}$ autophagosomes, which indicated that the inhibitory effect of

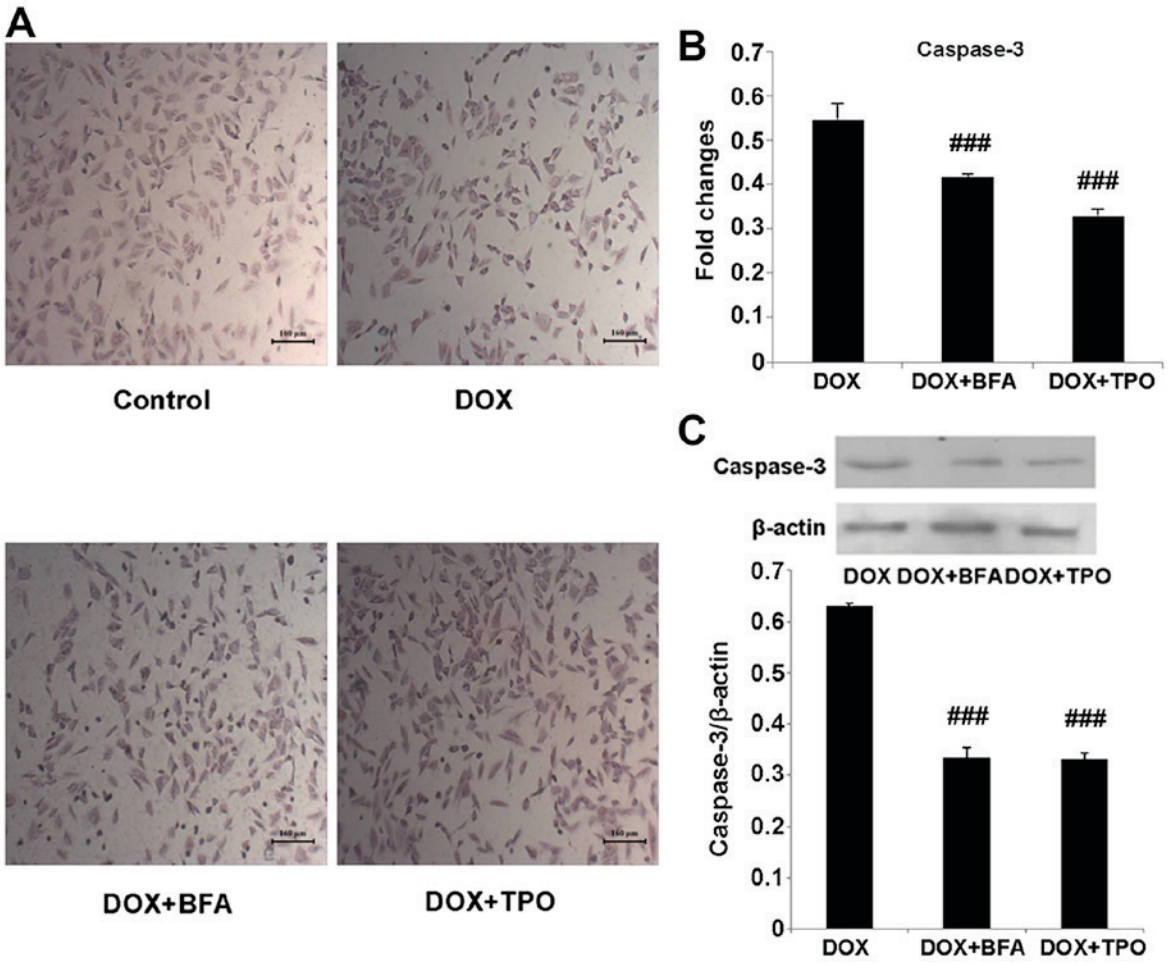

Figure 4. TPO reduced DOX-induced apoptosis in H9C2 cells. Grouping was set the same as described in Fig. 1. (A) Hematoxylin and eosin staining of H9C2 cells in each group. Scale bar, 160 μm. (B) Reverse transcription-quantitative polymerase chain reaction of genes encoding caspase-3. (C) Western blots: Representative images and quantification histograms for caspase-3 proteins. Results are presented as the mean ± standard deviation and were estimated by one-way analysis of variance with least significant difference post hoc test (n=3). $^{###}$P<0.001 vs. DOX treated alone group. DOX, doxorubicin; TPO, thrombopoietin; BFA, bafilomycin A1.

TPO on DOX-induced autophagy was irrelevant to autophagosome maturation arrest.

Furthermore, it was revealed that TPO downregulated the autophagy-promoting factor Beclin 1, while BFA brought about an opposite result of Beclin 1 regulation, which may be associated with feedback mechanisms for compensating the blocked autophagy flux. Taken together, these results indicated that TPO may negatively regulate autophagy at the early stage, which is different from the autophagy-inhibitory functions of BFA primarily in autolysosome formation.

It has been demonstrated that autophagy can promote survival by inhibiting apoptosis or facilitating apoptosis (51). The present study indicated that DOX induces autophagy and facilitates apoptosis in cardiomyocytes. GATA-4 is a zinc finger transcription factor, which protects cardiomyocytes from DOX-induced toxicity by inhibiting autophagy as well as apoptosis through regulating expression of Bcl-2 and Beclin1 (5). GATA-4 performs important roles in the development and hypertrophy of adult cardiac myocytes (33). Its downregulation is critical in DOX-induced cardiotoxicity (33). In the present study, the mRNA and protein level of GATA-4 and Bcl-2 were all significantly reduced in cardiomyocytes exposed to DOX, which was consistent with a previous study (5). Notably, TPO reversed the protein amount decrease. Additional studies are necessary to reveal the comprehensive roles of TPO in the cross talk between autophagy and apoptosis at the molecular level, particularly to investigate whether TPO inhibits autophagy and apoptosis of cardiomyocytes through regulating GATA-4 and/or Bcl-2 expression.

In conclusion, the present study demonstrated that DOX induces autophagy in cardiomyocytes, which contributes to DOX-induced cardiomyocyte death. TPO is able to attenuate DOX-induced cardiomyocyte autophagy and apoptosis. GATA-4 and Bcl-2 may be involved in the regulation of TPO on autophagy. This study investigated the new mechanism that the cardioprotective role of TPO on doxorubicin-induced myocardial damage and could provide a theoretical basis for TPO used as a cardioprotective agent in clinical.

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