

Original Article

Tauroursodeoxycholic acid inhibits endoplasmic reticulum stress, blocks mitochondrial permeability transition pore opening, and suppresses reperfusion injury through GSK-3 β in cardiac H9c2 cells

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Received June 2, 2016; Accepted October 30, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: This study investigates whether inhibition of endoplasmic reticulum (ER) stress prevents opening of the mitochondrial permeability transition pore (mPTP) and evaluates the corresponding signaling pathways involved in this process. Exposure of cardiac H9c2 cells to 800 μ M H₂O₂ for 20 min opened mPTP in response to oxidative stress, as demonstrated by quenching of tetramethylrhodamine ethyl ester (TMRE) fluorescence. Oxidative stress-induced mPTP opening was rescued by the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) in a dose-dependent manner at low concentrations. The PI3K and PKG inhibitors LY294002 and KT5823 inhibited the effect of TUDCA on mPTP opening, suggesting the involvement of PI3K/Akt and PKG signaling pathways. TUDCA significantly increased glycogen synthase kinase 3 (GSK-3 β) phosphorylation at Ser-9, with peak effect at 30 μ M TUDCA. The level of GRP78 (ER chaperone) expression was significantly upregulated by 30 μ M TUDCA. TUDCA-induced increases in Akt and GSK-3 β phosphorylation were inhibited by LY294002, whereas KT5823 suppressed TUDCA-induced increases in VASP and GSK-3 β phosphorylation. Oxidative stress severely affected cell morphology and ultrastructure. TUDCA prevented H₂O₂-induced ER swelling and mitochondrial damage. TUDCA boosted the viability of cells disrupted by ischemia/reperfusion (I/R), indicating that TUDCA eased reperfusion injury. However, TUDCA did not improve the viability of cells expressing the constitutively active GSK-3 β mutant (GSK-3 β -S9A-HA) that were subjected to I/R, suggesting an essential role of GSK-3 β inactivation in TUDCA-mediated cardioprotection against reperfusion damage. These data indicate that ER stress inhibition prevents mPTP opening and attenuates reperfusion injury through GSK-3 β inactivation. The PI3K/Akt and PKG pathways may mediate GSK-3 β inactivation.

Keywords: ER stress, TUDCA, mPTP, GSK-3 β , reperfusion injury

Introduction

The endoplasmic reticulum (ER) is a crucial cellular organelle for protein synthesis, protein topology, maintenance of Ca²⁺ homeostasis, and biosynthesis of lipids and steroids [1-3]. Disruption of the internal ER microenvironment leads to ER dysfunction [4], which is designated as ER stress. ER stress is involved in numerous pathophysiological processes, including aging, inflammation, diabetes, neurodegenerative diseases, and cardiovascular diseases [5]. ER and mitochondria interact closely with each other, and sustained ER stress can induce mitochondrial stress [7, 8].

The mitochondrial permeability transition pore (mPTP) has a critical role in the pathogenesis of cardiac ischemia/reperfusion (I/R) injury. Regulation of mPTP opening is proposed to be the common effector of myocardial protection against I/R injury [9-11]. Inhibition of ER stress attenuates mPTP opening and promotes cardioprotection against I/R injury, although the mechanism has not been elucidated. In this study, we investigate whether blocking mPTP opening protects heart cells against reperfusion injury [12-14].

Glycogen synthase kinase 3 (GSK-3 β) occupies an integration point for several signaling path-

ways, and has a critical role in transferring protective signals downstream to mPTP targets [15]. ER stress-induced apoptosis involves the loss of mitochondrial membrane potential ($\Delta\Psi_m$), which is directly caused by mPTP opening [16]. GSK-3 β is the direct upstream regulator of mPTP15. It is reasonable to speculate that inhibition of ER stress may block mPTP opening by inactivating GSK-3 β .

In this study, we tested whether the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) blocks mPTP opening and attenuates reperfusion injury in cardiac H9c2 cells. We also investigated the molecular mechanism underlying TUDCA action, focusing on potential roles in PI3K/Akt, PKG, and GSK-3 β signaling pathways.

Materials and methods

Cell culture

The cardiac myoblast H9c2 cell line (derived from rat heart tissue) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Chemicals and antibodies

TUDCA, LY294002, and KT5823 were obtained from Sigma (St. Louis, MO, USA). All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Tetramethylrhodamine ethyl ester (TMRE) was obtained from Molecular Probes (Eugene, OR, USA).

GSK-3 plasmid DNA

The following two GSK-3 β mutant plasmids containing HA tags were kindly provided by Dr. Morris Birnbaun (University of Pennsylvania School of Medicine): catalytically inactive GSK-3 β (GSK-3 β -KM-HA) and constitutively active GSK-3 β (GSK-3 β -S9A-HA). Plasmid DNA was purified with the Endofree Maxi kit (Qiagen). Then, 2 μ g of plasmid DNA was used to transiently transfect H9c2 cells in 12-well plates [16]. Briefly, H9c2 cells were seeded in a 12-well plate at 50% confluence and allowed to grow for 2 h. Then, cells were transfected with

β -galactosidase (pCDNA-His-LacZ) or the GSK-3 β mutants using Fugene 6 transfection reagents according to the manufacturer's instructions (DNA: reagent = 1:3; Roche). Cells were replenished with fresh medium 24 h after transfection. Experiments with transfected cells were conducted 48 h after transfection. The transfection efficiency was tested using the β -galactosidase assay kit (Invitrogen). More than 80% of cells routinely expressed β -galactosidase (> 80% transfection; data not shown).

Confocal imaging of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by loading H9c2 cardiac cells with TMRE and visualizing with confocal microscopy as reported previously [17]. Cells were cultured in a specific temperature-controlled culture dish (MatTek, MA, USA), then incubated with TMRE (100 nM) in standard Tyrode solution (140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES pH 7.4, and 5.8 mM glucose) for 10 min. Labeled cells were mounted on the stage of an Olympus FLUOVIEW FV 1000 laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan). Red fluorescence was excited with 543 nm He-Ne laser line and imaged through a 560 nm long-path filter. Temperature was maintained at 37°C. The images were quantified with ImageJ software (NIH, USA).

Western blotting analysis

Equal amounts of protein lysates were electrophoresed on an SDS-polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed overnight at 4°C with primary antibodies (1:1000) that recognize the phosphate moieties of GSK-3 β , vasodilator-stimulated phosphoprotein (VASP), and Akt. After washing, the membrane was probed with corresponding secondary antibodies (1:1000). Finally, the membrane was visualized using enhanced chemiluminescence (ECL) method and images were captured using the Biospectrum Imaging System (UVP, Upland, USA). Equal protein loading was verified by reprobing the membrane with antibodies that recognize total GSK-3 β , Akt, or tubulin.

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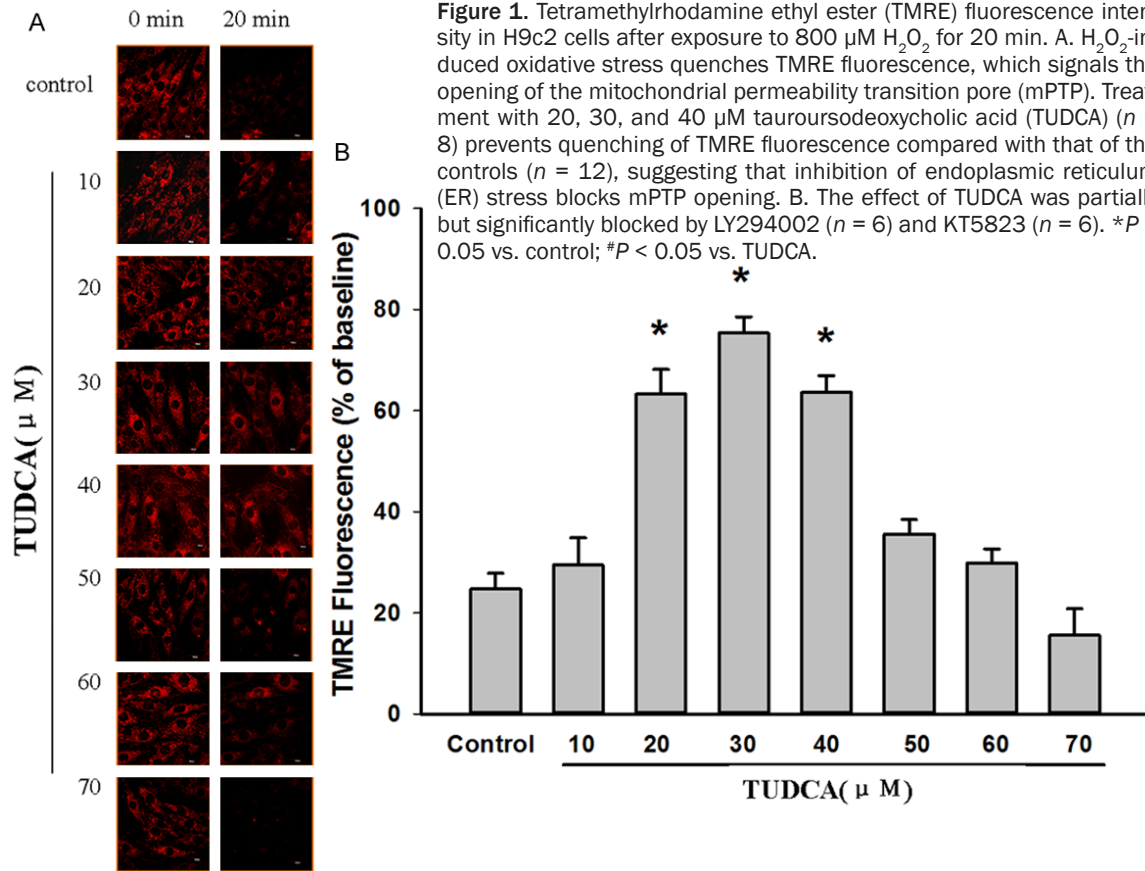


Figure 1. Tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity in H9c2 cells after exposure to 800 μM H_2O_2 for 20 min. A. H_2O_2 -induced oxidative stress quenches TMRE fluorescence, which signals the opening of the mitochondrial permeability transition pore (mPTP). Treatment with 20, 30, and 40 μM tauroursodeoxycholic acid (TUDCA) ($n = 8$) prevents quenching of TMRE fluorescence compared with that of the controls ($n = 12$), suggesting that inhibition of endoplasmic reticulum (ER) stress blocks mPTP opening. B. The effect of TUDCA was partially but significantly blocked by LY294002 ($n = 6$) and KT5823 ($n = 6$). * $P < 0.05$ vs. control; # $P < 0.05$ vs. TUDCA.

Flow cytometry assay of cell viability

Cell viability was assessed by propidium iodide fluorometry using a flow cytometer (FACScalibur, Becton Dickinson, NJ). Fluorescence intensity was measured at the excitation and emission wavelengths of 488 and 590 nm, respectively. Cells were incubated in standard Tyrode solution for 2 h before the experiments. Cells were then subjected to 90 min of simulated ischemia followed by 30 min of reperfusion.

Transmission electron microscopy of ER and mitochondria

Cells were fixed with 2.5% glutaraldehyde (Fluka Analytical, Sigma) in phosphate buffer at 4°C for 24 h. Cells were post-fixed with 0.1% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA) in water for 1 h, dehydrated through a graded ethanol series, and then embedded in araldite M (Fluka Analytical, Sigma). Sections (70-80 nm) were observed using the Hitachi H7500 transmission electron microscope.

Experimental protocols

Cultured cells were washed twice with phosphate buffered saline (PBS) and then incubated in standard Tyrode solution for 2 h before experiments. To test the effects of TUDCA on GSK-3 β phosphorylation at Ser-9, VASP phosphorylation at Ser-239, or Akt phosphorylation at Ser-473, cells were exposed to different concentrations of TUDCA (10-70 μM) for 20 min. Inhibitors were applied 10 min before exposure to TUDCA. The effect of TUDCA on $\Delta\Psi_m$ was evaluated. Cells were exposed to 800 μM H_2O_2 for 20 min to induce mitochondrial oxidative damage. Cells were treated with TUDCA 20 min before treatment with H_2O_2 .

Statistical analysis

Data were obtained from at least six experiments and are expressed as mean \pm SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's test. A value of $P < 0.05$ was considered as statistically significant.

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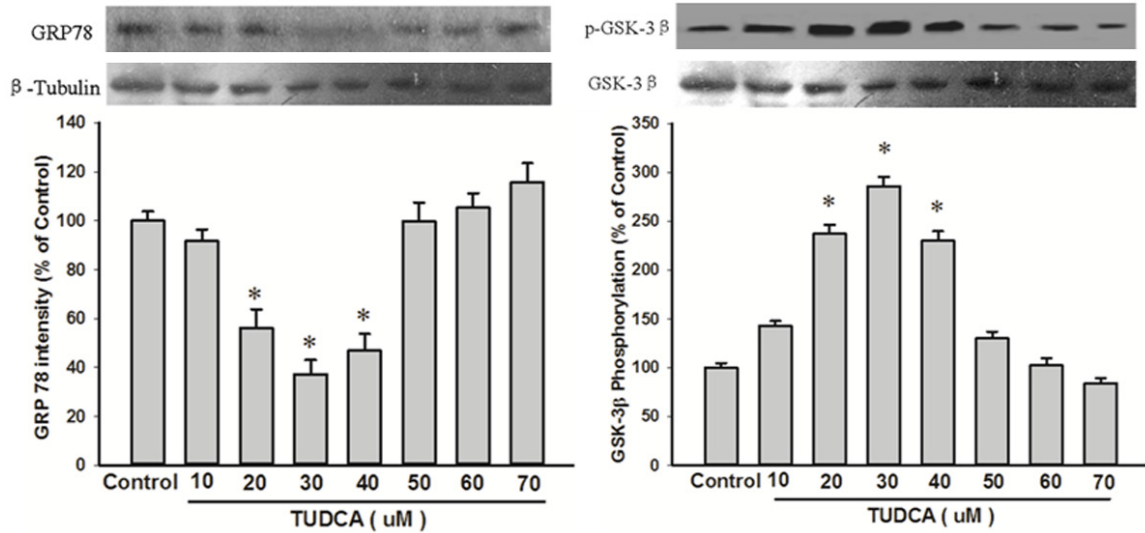


Figure 2. Western blot analysis of phospho-GSK-3 β (Ser-9) and GRP 78 in cardiac H9c2 cells. TUDCA ($n = 8$) significantly increased GSK-3 β phosphorylation at Ser-9, with the peak effect at 30 μ M TUDCA. Expression of the ER stress marker GRP 78 also peaks at 30 μ M TUDCA ($n = 8$), indicating that TUDCA inhibits ER stress, and inhibition of ER stress inactivates GSK-3 β . * $P < 0.05$ vs. control.

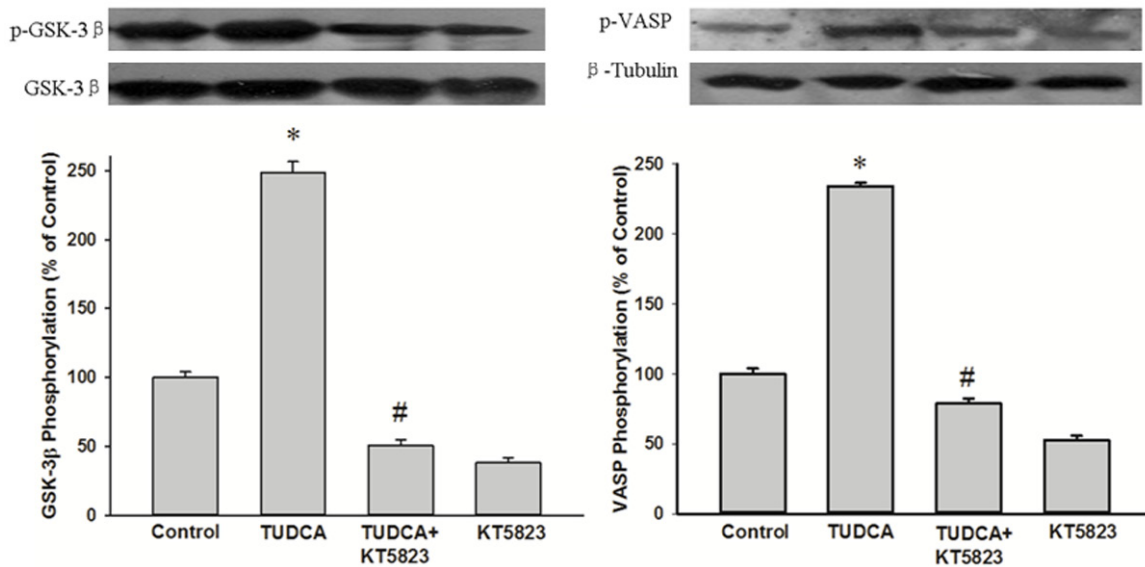


Figure 3. Western blot analyses of phosphorylation of GSK-3 β (Ser-9) and vasodilator-stimulated phosphoprotein (VASP) in cardiac H9c2 cells. KT5823 ($n = 6$) suppressed TUDCA-mediated phosphorylation ($n = 8$) of VASP and GSK-3 β , indicating that PKG may mediate the action of TUDCA. * $P < 0.05$ vs. control; # $P < 0.05$ vs. TUDCA.

Results

First, we defined the effect of ER stress on mPTP opening by testing whether TUDCA (the classic ER stress inhibitor) prevented oxidative stress-induced loss of $\Delta\Psi_m$, which was detected by monitoring changes in TMRE fluorescence using confocal microscopy. Cells were loaded with TMRE and then treated with 800

μ M H_2O_2 for 20 min, which induced the quenching of TMRE fluorescence ($25.4 \pm 3.4\%$ of baseline in the control group) (Figure 1). This indicates that mPTP opening is induced by oxidative stress. By contrast, cells treated with 20, 30, and 40 μ M TUDCA were protected from TMRE fluorescence quenching ($63.2 \pm 4.8\%$, $75.3 \pm 2.7\%$, and $63.6 \pm 2.4\%$ of baseline in the control group, respectively) (Figure 1). The peak

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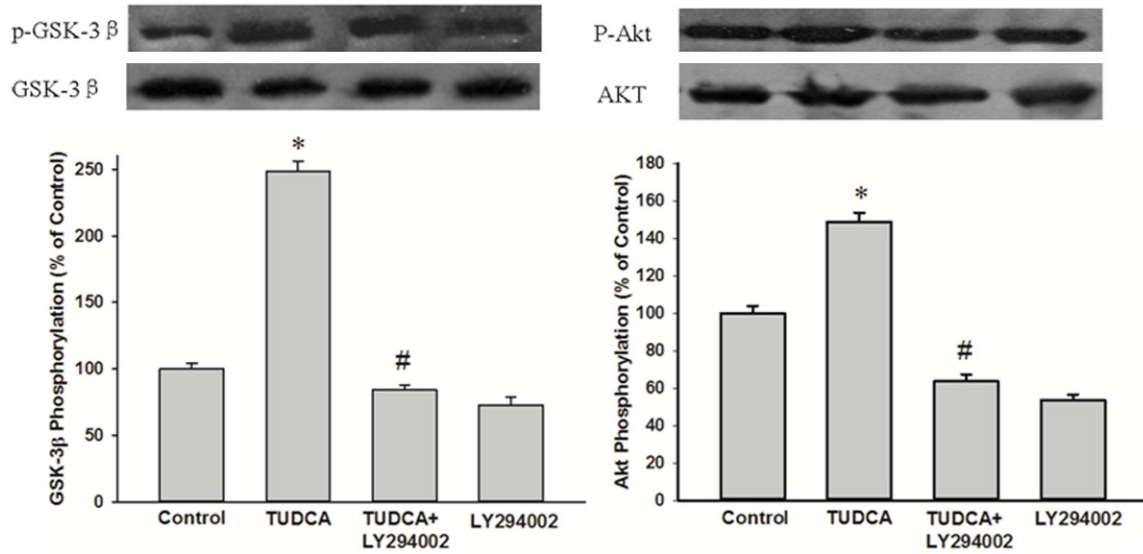


Figure 4. Western blot analyses of phosphorylation of GSK-3 β (Ser-9) and Akt in cardiac H9c2 cells. LY294002 ($n = 6$) suppressed TUDCA-mediated phosphorylation of Akt and GSK-3 β , indicating that PI3K/Akt may mediate the action of TUDCA. * $P < 0.05$ vs. control; # $P < 0.05$ vs. TUDCA.

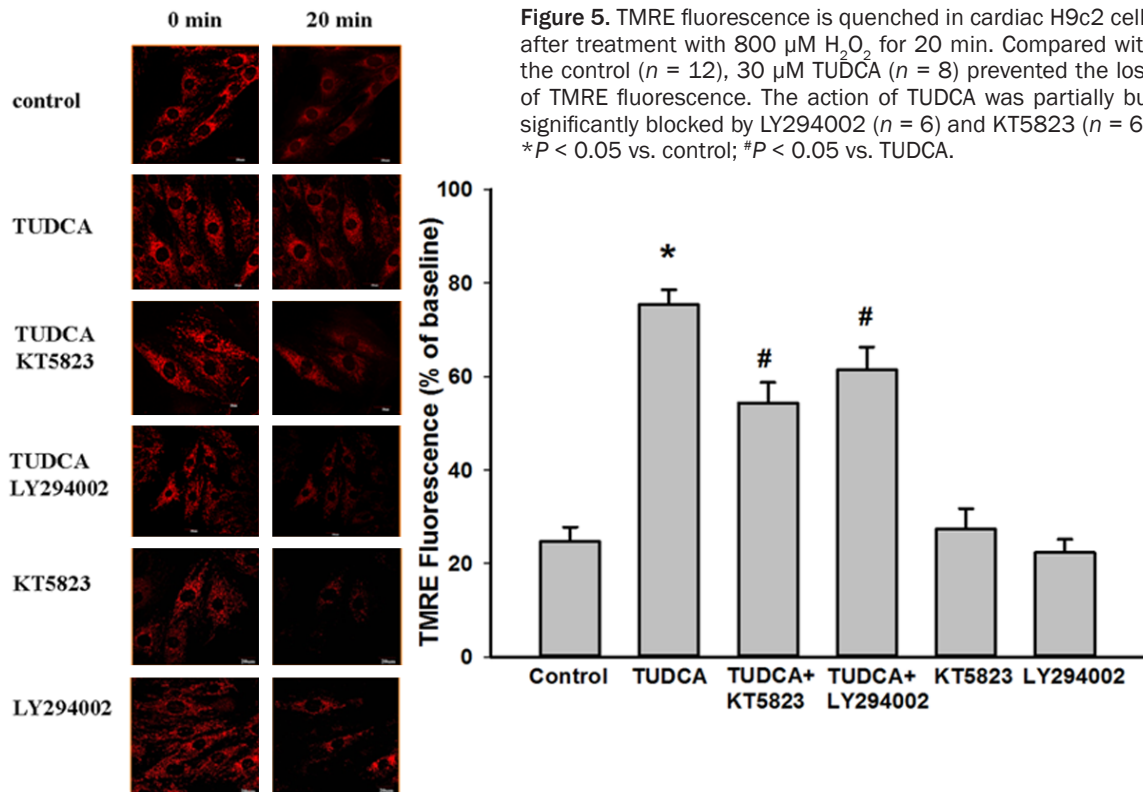


Figure 5. TMRE fluorescence is quenched in cardiac H9c2 cells after treatment with 800 μM H_2O_2 for 20 min. Compared with the control ($n = 12$), 30 μM TUDCA ($n = 8$) prevented the loss of TMRE fluorescence. The action of TUDCA was partially but significantly blocked by LY294002 ($n = 6$) and KT5823 ($n = 6$). * $P < 0.05$ vs. control; # $P < 0.05$ vs. TUDCA.

protective effect was conferred by 30 μM TUDCA. These results suggest that inhibition of ER stress blocks mPTP opening.

To test whether inhibition of ER stress inactivates GSK-3 β in H9c2 cells, we determined the

effect of TUDCA on GSK-3 β phosphorylation at Ser-9 (Figure 2). TUDCA significantly increased GSK-3 β phosphorylation at Ser-9 in a dose-dependent manner, with the peak effect at 30 μM TUDCA ($285.6 \pm 9.9\%$). The highest level of GRP78 expression also was observed at 30 μM

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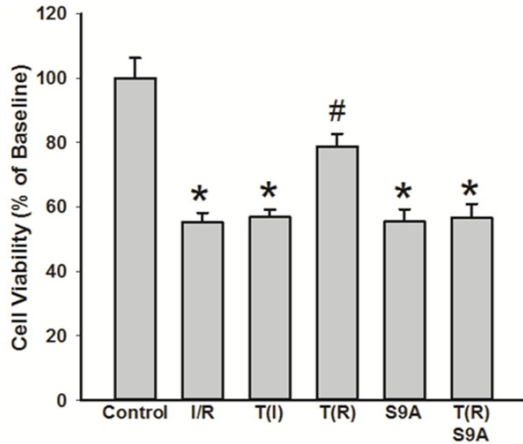


Figure 6. Cell viability analysis of H9c2 cells using flow cytometry. Cells were treated with TUDCA ($n = 8$) at the onset of reperfusion [T(R)], which improved cell viability compared with cells subjected to ischemia followed by reperfusion. Treatment of cells with TUDCA ($n = 6$) at reperfusion failed to improve the viability of cells transfected with constitutively active GSK-3 β (S9A), indicating that TUDCA prevents reperfusion injury through inactivation of GSK-3 β . * $P < 0.05$ vs. control; # $P < 0.05$ vs. I/R. I, ischemia; R, reperfusion.

TUDCA ($37.3 \pm 5.7\%$). These results suggest that TUDCA inhibits ER stress by inactivating GSK-3 β .

To explore the mechanism of TUDCA-induced GSK-3 β inactivation, we tested whether the PI3K inhibitor LY294002 and the PKG inhibitor KT5823 altered TUDCA-mediated inhibition of ER stress and mPTP opening. The effect of TUDCA on GSK-3 β phosphorylation was partially, but significantly, inhibited by LY294002 and KT5823 (**Figures 3 and 4**). LY294002 inhibited TUDCA-induced increases in Akt and GSK-3 β phosphorylation, whereas KT5823 inhibited TUDCA-induced increases in VASP and GSK-3 β phosphorylation. These results suggest that the PI3K/Akt and PKG signaling pathways are involved in TUDCA-induced GSK-3 β inactivation (**Figures 3 and 4**). LY294002 and KT5823 also inhibited TUDCA-mediated protection of TMRE fluorescence quenching by oxidative stress (**Figure 5**). These combined results strongly support the hypothesis that the mechanism of TUDCA-mediated inhibition of mPTP opening involves the PI3K/Akt and PKG signaling pathways via GSK-3 β .

GSK-3 β has an important role in cardioprotection, suggesting that inhibition of ER stress may confer cardioprotection against ischemia/

reperfusion injury via a GSK-3 β -dependent mechanism. To test the effect of TUDCA on ischemia/reperfusion injury, H9c2 cells were subjected to 90 min of simulated ischemia followed by 30 min of reperfusion. Simulated ischemia/reperfusion significantly reduced cell viability to $55.3 \pm 2.9\%$ compared with that of control cells (**Figure 6**). Treatment of cells with TUDCA during ischemia, but not during reperfusion, failed to improve cell viability ($55.1 \pm 3.2\%$). By contrast, treatment of cells with TUDCA at the onset of reperfusion for 30 min dramatically increased cell viability to $78.8 \pm 3.9\%$, indicating that TUDCA has cardioprotective activity during reperfusion rather than during ischemia. Therefore, TUDCA may protect the myocardium from reperfusion injury. To examine whether the protective effect of TUDCA was mediated by GSK-3 β inhibition, cells were transfected with the constitutively active GSK-3 β mutant (GSK-3 β -S9A), and then were treated with TUDCA at the onset of reperfusion. The results showed that TUDCA did not improve cell viability ($51.2 \pm 3.5\%$) in cells transfected with the constitutively active GSK-3 β mutant (GSK-3 β -S9A-HA; **Figure 6**). These combined results strongly support an essential role of GSK-3 β inactivation in cardioprotection conferred by inhibition of ER stress.

We analyzed the effects of TUDCA on subcellular ER and mitochondrial morphologies using transmission electron microscopy (**Figure 7**). Treatment of cells with $800 \mu\text{M}$ H_2O_2 induced marked morphological changes and organellar damage, including ER swelling, mitochondrial vacuolization, and loss of mitochondrial cristae. By contrast, TUDCA alleviated H_2O_2 -induced ultrastructural abnormalities, suggesting that TUDCA-mediated inhibition of ER stress may prevent oxidation-induced mitochondrial damage (**Figure 7**).

Discussion

This study showed that TUDCA-mediated inhibition of ER stress blocked mPTP opening and protected cardiac H9c2 cells against reperfusion injury. Our results indicate that TUDCA action is transduced through PI3K/Akt and PKG signaling-mediated inactivation of GSK-3 β .

Disruption of ER function leads to ER stress via activation of complex cytoplasmic and nuclear signaling pathways. This is collectively termed

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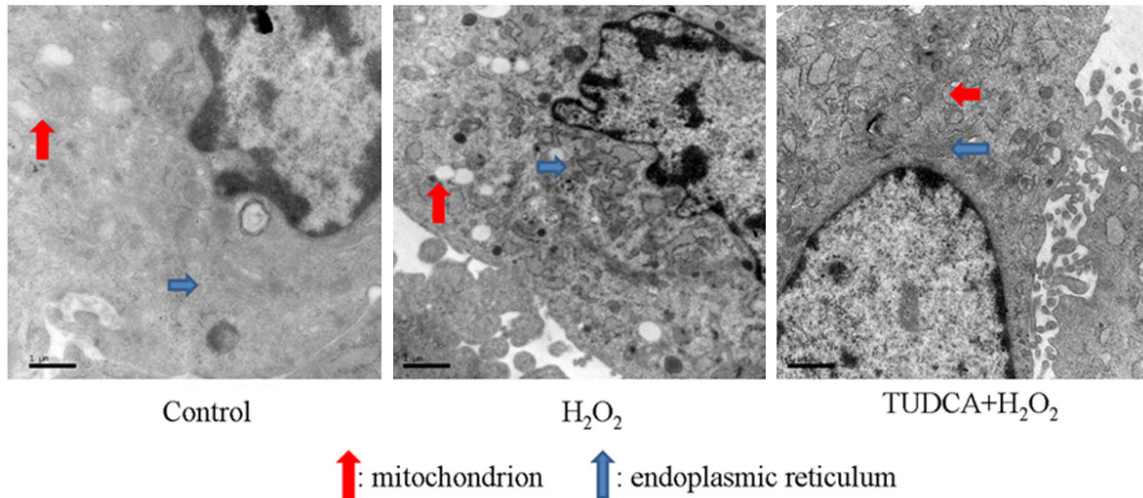


Figure 7. Transmission electron microscopy analysis of subcellular structures in cells subjected to oxidative stress. TUDCA prevented H_2O_2 -induced swelling of ER and mitochondria, suggesting that inhibition of ER stress prevents oxidant-induced cellular damage. Red arrows indicate mitochondria; blue arrows indicate ER. Total magnification, $\times 23,853$; indicated magnification, $\times 12,000$.

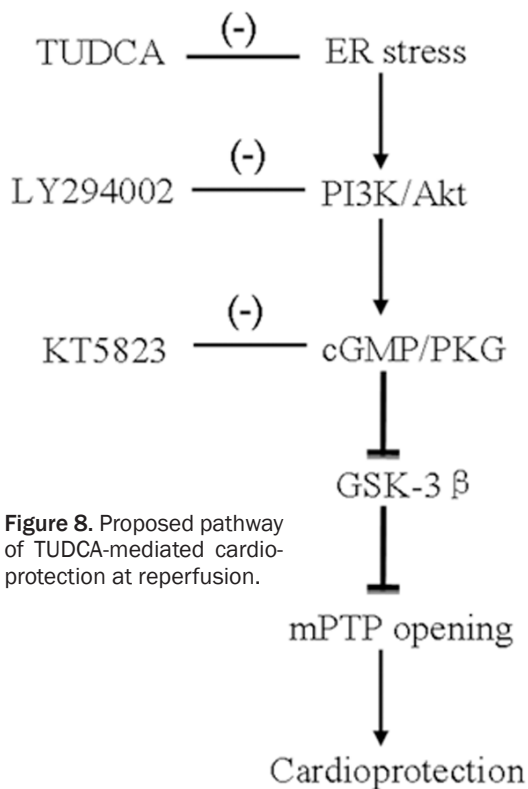


Figure 8. Proposed pathway of TUDCA-mediated cardioprotection at reperfusion.

regarded as a classic ER stress chaperone. ER stress is involved in many pathophysiological processes, including aging, inflammation, diabetes, neurodegenerative diseases, and cardiovascular diseases [5]. Early studies demonstrated the existence of a functional ER compartment in cardiomyocytes [21]. Recent reports show that ER-associated functions have critical roles in cardiac pathophysiology [22, 23]. Calcium overload or simulated ischemia significantly increased expression of the ER chaperone GRP 94. Regulation of GRP94 by XBP1 and ATF6 attenuates cardiomyocyte necrosis [24]. Hypoxia increases the levels of XBP1 mRNA transcription and promotes GRP 78 protein translation [25]. Ischemic preconditioning or postconditioning reduces cardiac damage associated with UPR activation [26, 27]. ER stress is involved in several cardiovascular diseases, such as cardiac hypertrophy, heart failure, atherosclerosis, and ischemic heart disease [28-33]. ER stress is associated with cardiomyocyte apoptosis [34, 35] and the loss of cell viability following ischemia/reperfusion [36, 37]. ER stress represents a therapeutic target for a number of cardioprotective signaling molecules such as AMP-activated protein kinase (AMPK) [32]. AMPK remediates ER stress through eEF2 inactivation, which preserves sarco (endo) plasmic reticulum function and intracellular Ca^{2+} homeostasis [32, 38]. These observations suggest that inhibition of

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ER stress has crucial roles in cardioprotection and the prevention of reperfusion injury.

The ER stress inhibitor TUDCA reduced ER stress in the hearts of type 2 diabetic rats, thereby blocking mPTP opening through GSK-3 β [8]. TUDCA is the taurine-conjugated form of ursodeoxycholic acid (UDCA), which is an endogenous bile acid. TUDCA is detected in human bile, and has a long history in Chinese medicine as a remedy for biliary and liver diseases. TUDCA exerts anti-apoptotic effects and enhances cardiac function through the Bcl-2 family and survival signaling proteins such as Bax, Bad, and PI3-K. TUDCA treatment reduces infarct size in mice that lost cyclophilin D (cypD) function [39, 40]. Treatment of obese and diabetic mice with TUDCA reversed hyperglycemia and insulin sensitivity, reduced fatty liver, and enhanced the action of insulin in the liver, muscle, and adipose tissues [41]. Thus, accumulating evidence indicates that inhibition of ER stress is crucial for cardioprotection. We propose that TUDCA-mediated inhibition of ER stress blocks mPTP opening and reperfusion injury through the inactivation of GSK-3 β .

The opening of mPTP is a critical determinant of myocardial ischemia/reperfusion injury [42], and mPTP is an important target for cardioprotective factors [43]. Preconditioning and postconditioning confer cardioprotection against ischemia/reperfusion injury by inhibiting the opening of mPTP [44-46]. The present study shows that treatment of cardiac cells with 800 μM H_2O_2 quenched TMRE fluorescence, suggesting that oxidative stress leads to the loss of $\Delta\Psi_m$, which is also known to result from mPTP opening. Treatment with TUDCA reduced oxidative stress-induced TMRE fluorescence quenching, indicating that inhibition of ER stress blocked oxidative stress-induced mPTP opening. Under our experimental conditions, 30 μM TUDCA conferred optimal cardioprotection.

The exact signaling pathway involved in blocking mPTP opening has not been completely elucidated, although the available evidence suggests that GSK-3 β mediates this cardioprotective process [11]. GSK-3 β is inactivated by phosphorylation at Ser-9 and Tyr-216. Phosphorylation at Ser-9 decreases the activity of GSK-3 β , whereas phosphorylation at Tyr-216 increases GSK-3 β activity [24]. GSK-3 β is constitutively activated by basal phosphorylation

of Tyr-216. GSK-3 β inactivation has a critical role in modulating cardioprotective effects under ischemic preconditioning [47], morphine [48], and bradykinin [49]. GSK-3 β modulates cardioprotective signaling pathways by inhibiting the opening of mPTP [46]. Consistently, preconditioning [50] and postconditioning [51] prevent the opening of mPTP by inactivating GSK-3 β . The present study shows that TUDCA significantly increases GSK-3 β phosphorylation at Ser-9 in a dose-dependent manner, with the peak effect conferred at 30 μM TUDCA. These results suggest that inhibition of ER stress inactivates GSK-3 β in cardiac cells. The ER chaperone GRP78 is strongly downregulated by treatment with 30 μM TUDCA, indicating that TUDCA inhibits ER stress.

The PI3K/Akt-PKG-GSK-3 β signaling pathway is essential for cardioprotection, and has been proposed to prevent mPTP opening [52-54]. The effect of TUDCA on GSK-3 β phosphorylation can be reversed by the PI3K/Akt inhibitor LY294002 and the PKG inhibitor KT5823, indicating that the PI3K/Akt and PKG signaling pathways are required for the inhibitory action of TUDCA. TUDCA significantly increases the phosphorylation of Akt at Ser-473 and VASP at Ser-216, which is a substrate of PKG. These effects also are inhibited by LY294002 and KT5823, which further confirms that TUDCA inactivates GSK-3 β via its upstream PI3K/Akt and PKG pathways. We show that TUDCA-mediated protection of oxidative stress-induced TMRE fluorescence quenching is reversed by LY294002 and KT5823, suggesting that the PI3K/Akt and PKG pathways are involved in this process. By contrast, TUDCA did not block TMRE fluorescence quenching under oxidative stress in cells transfected with the constitutively active GSK-3 β mutant (GSK-3 β -S9A). This result strongly supports the essential role of GSK-3 β inactivation in cardioprotection. A reactive oxygen burst during myocardial reperfusion is associated with cardiac injury [55]. Therefore, our results suggest that inhibition of ER stress may protect the heart from reperfusion injury by modulating mPTP opening. This result is consistent with previous proposals that blockade of mPTP opening is a crucial target for cardioprotective interventions [56, 57].

GSK-3 β inactivation is an important mechanism for cardioprotection mediated by ischemic preconditioning or by some cardioprotectants

applied at reperfusion. GSK-3 β inactivation is proposed to protect the myocardium by inhibiting mPTP opening [11]. Many cardioprotective interventions against mPTP opening [59] are applied during reperfusion because reperfusion induces mPTP opening more strongly than ischemia [58]. GSK-3 β inactivation is crucial for cardioprotection during reperfusion. The present study shows that TUDCA protects cardiac cells during reoxygenation but not during ischemia, suggesting that cardioprotection induced by inhibition of ER stress occurs during reperfusion. These results also suggest the therapeutic potential of inhibiting ER stress during coronary occlusion induced by acute myocardial infarction. Our results show that TMRE fluorescence is quenched during reperfusion of constitutively active GSK-3 β mutant cells (GSK-3 β -S9A) even in the presence of TUDCA, and TUDCA treatment induced GSK-3 β phosphorylation at Ser-9 (inactivation), thereby inactivating the GSK-3 β pathway. These combined results suggest that inhibition of ER stress protects cells from reperfusion injury by inactivating GSK-3 β . We also observe that catalytically inactive GSK-3 β mutant cells (GSK-3 β -KM) have similar levels of cardioprotection with or without TUDCA treatment, which confirms the importance of GSK-3 β inactivation for protection against ischemia/reperfusion injury.

We observed that H₂O₂-induced oxidative stress caused drastic morphological changes and organellar damage, such as ER swelling, mitochondrial vacuolization, and loss of mitochondrial cristae. TUDCA treatment effectively alleviated H₂O₂-induced ultrastructural abnormalities, suggesting that inhibition of ER stress protects cells from oxidant-induced damage.

We elected to investigate the protective effect of TUDCA in cardiac H9c2 cells rather than in cardiomyocytes, perfused hearts, or sentient animals. Although the H9c2 cell line is generally accepted as a suitable model for cardiomyocytes, it is not fully differentiated into cardiomyocytes [60, 61]. Further studies are needed to verify these results during myocardial ischemia/reperfusion.

In conclusion, our data indicate that TUDCA inhibits ER stress and reperfusion injury by preventing mPTP opening via GSK-3 β inactivation and the PI3K/Akt and PKG pathways (**Figure 8**).

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81570275), China Postdoctoral Science Foundation (2014 M560190), the Research Project of Education Department of Hebei Province (ZD 2014006), North China University of Science and Technology Foundation for Outstanding Young Scholars (JP 201302).

Disclosure of conflict of interest

None.

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