Pterostilbene attenuates myocardial ischemia-reperfusion injury via the phosphatidylinositol 3'-kinase-protein kinase B signaling pathway

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Received October 27, 2016; Accepted June 23, 2017

DOI: 10.3892/etm.2017.5246

Abstract. The current study aimed to evaluate the cardioprotective effects of pterostilbene (PTB) on myocardial ischemia-reperfusion (I/R) injury in rats and identify its possible underlying mechanisms of action. A rat I/R model was established by ligating the left anterior descending coronary artery for 30 min and releasing the ligature to induce reperfusion for 120 min. Serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) levels were measured using CK-MB and LDH assay kits and myeloperoxidase (MPO) activity in the myocardium was evaluated using an MPO assay kit. Tumor necrosis factor-α, interleukin (IL)-6 and IL-8 levels were assayed using ELISA kits. Cardiomyocyte apoptosis was measured using terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Levels of protein kinase B (Akt) and phosphorylated Akt (p-Akt) were measured using western blotting. The results demonstrated that treatment with PTB significantly reduced cardiomyocyte apoptosis, significantly increased Bcl-2 and p-Akt levels and decreased Bax expression in the hearts of rats subjected to I/R injury. However, the protective effects induced by PTB were attenuated by LY294002, which inhibits Akt activation. The results of the current study suggest that PTB treatment may reduce the I/R injury-induced apoptosis of cardiomyocytes, which is mediated by the phosphoinositide 3-kinase/Akt signaling pathway.

Introduction

Myocardial infarction is a major cause of mortality and morbidity (1). Currently the main method of treating myocardial infarction is the re-establishment of blood flow as early as possible to prevent further injury to the myocardium. However, this may cause myocardial ischemia/reperfusion (I/R) injury and there are currently no therapeutic strategies available to treat this (2). Myocardial I/R injury induces a series of pathological changes, including cellular apoptosis, calcium overload and oxidative stress (3,4). Therefore, it is critical to identify novel therapeutic agents to reduce I/R injury in patients with myocardial infarction.

Pterostilbene (PTB), a natural phytoalexin found in blueberries, is a dimethylated analog of resveratrol (5). Previous studies have demonstrated that PTB exhibits various biological roles, including anti-inflammatory, anti-oxidation and anti-apoptotic functions (6-8). Oxidative stress, apoptosis and inflammation serve critical roles in myocardial I/R injury; thus, PTB is a promising candidate for the treatment of myocardial I/R injury. However, the effects of PTB on myocardial I/R injury remain unclear.

The phosphatidylinositol 3'-kinase-protein kinase B (PI3K/Akt) signaling pathway co-ordinates various intracellular processes, including regulation of cell proliferation and survival (9,10). The PI3K/Akt signaling pathway is a pro-survival signaling pathway that confers protection against myocardial ischemia (11). However, it remains unknown whether PTB confers cardioprotection via activation of the PI3K/Akt signaling pathway. Therefore, the current study aimed to determine whether PTB reduces myocardial I/R injury and if so, identify whether PI3K/Akt signaling is activated by treatment with PTB.

Materials and methods

Animals. A total of 60 adult male Sprague-Dawley rats (250-300 g; 1.5-2 months) were obtained from the Center of Experimental Animals in the Xi'an Jiaotong University (Xi'an, China). Animals were housed in a controlled room with a temperature of 22˚C, 33% humidity, a 12 h light/dark cycle and free access to food and water. All animals used in the current study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council (US) Committee (12) and all procedures

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Key words: pterostilbene, phosphoinositide 3-kinase/protein kinase B signaling, myocardial ischemia-reperfusion injury

were approved by The Committee of Experimental Animals of the Xi'an Jiaotong University.

Reagents. PTB, the PI3K inhibitor LY294002 and DAPI were all purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Myocardial myeloperoxidase (MPO; cat. no. A044), creatine kinase-MB (CK-MB; cat. no. H197) and lactate dehydrogenase (LDH; cat. no. A020-1) assay kits were all purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Interleukin-6 (IL-6; cat. no. R6000B) and tumor necrosis factor-α (TNF-α; cat. no. RTA00) ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). IL-8 ELISA kit (cat. no. kt30449) was purchased from MSK Bio (Wuhan, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kits were purchased from Roche Applied Science (Mannheim, Germany). Antibodies against Akt (cat. no. 4685), p-Akt (Ser473; cat. no. 4058), Bcl-2 (cat. no. 3498), Bax (cat. no. 14796) and β-actin (cat. no. 4970) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The horseradish peroxidase-conjugated immunoglobulin G secondary antibody (cat. no. A0208) was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Myocardial ischemia-reperfusion (MI/R) model and experimental protocol. Sprague-Dawley rats were anesthetized intraperitoneally (i.p.) using sodium pentobarbital (Sigma-Aldrich; Merck KGaA; 40 mg/kg). Myocardial ischemia was induced by exteriorizing the heart with a left thoracic incision followed by a slipknot (5-0 silk) around the left anterior descending coronary artery (LAD). Following 30 min ischemia, the slipknot was released and the animal underwent 120 min reperfusion. The Sham group underwent the same procedure without LAD ligation.

Rats were randomly assigned to one of four groups (n=15): i) A sham group; ii) an MI/R group that received treatment vehicle with 0.9% sodium chloride i.p.; iii) an MI/R+PTB group that received PTB (10 mg/kg, i.p.) 10 min prior to reperfusion iv) and an MI/R+PTB+LY group that received PTB (10 mg/kg, i.p.) 10 min prior to reperfusion and LY (10 mg/kg, i.p.) every 2 days three times prior to surgery.

Evaluation of myocardial infarct size. Tetrazolium chloride (TTC) staining was used to assess the myocardial infarct size. Following reperfusion, rats were anaesthetized and euthanized. Rat hearts were then immediately isolated, washed in PBS and sectioned into transverse slices 5 mm thick. Following incubation in 1% (0.01 g/ml) TTC at 37˚C in PBS for 15 min, heart slices were photographed with a digital camera to distinguish between the red-stained viable tissues and the white-unstained infarcted tissues. Areas of infarct size were measured digitally using Image Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Evaluation of serum levels of CK-MB and LDH. Following reperfusion, blood (6 ml) was taken from the carotid artery of all rats and kept at room temperature for 30 min. Serum was collected by centrifugation at 3,000 x g at 4 ˚C for 15 min and kept at -70˚C for preservation. The CK-MB and LDH assay kits were used to measure levels of CK-MB and LDH in the serum, following the manufacturer's instructions. Enzyme activity was expressed in U/l.

Assay of MPO activity and levels of the inflammatory cytokines TNF-α, IL-6, IL-8 in the serum. Following reperfusion, the myocardial tissue was kept at -70˚C for preservation. An MPO assay kit was used to detect MPO levels in the myocardial tissue following the manufacturer's instructions. Levels of TNF-α, IL-6 and IL-8 were spectrophotometrically analyzed using ELISA kits following the manufacturer's instructions.

Determination of myocardial apoptosis. Myocardial apoptosis was determined using TUNEL staining. The tissue samples were fixed using 4% formaldehyde at 4˚C for, 24 h and embedded in paraffin. The paraffin-embedded tissue was cut into sections 4-5 μm thick. Sections were incubated in 50 μl TUNEL mixture (solution A: Solution B=1:9) in a humidified chamber at 37˚C for 1 h. Slides were incubated with DAPI for 5 min at room temperature in the dark to detect the nuclei. Sections were covered using cover slips with anti-fade mounting medium (cat. no. P0126; Beyotime Institute of Biotechnology, Haimen, China). Sections were observed using fluorescence microscopy in six random fields of view. The apoptotic index was calculated as the ratio of the number of TUNEL-positive neurons to the total number of nuclei.

Western blot analysis. Heart tissues were lysed with RIPA lysis buffer (cat. no. sc-24948, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following sonication at 4˚C for 25 sec at a frequency of 20 kHz, lysates were centrifuged at 14,000 x g at 4˚C for 30 min and protein concentration was detected using the BCA determination method. Proteins (30 μg/lane) were then separated by SDS-PAGE (8-15%) and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% skimmed milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated with primary antibodies against p-Akt (1:1,000), Akt (1:1,000), Bcl-2 (1:1,000), Bax (1:1,000) and β-actin (1:1,000) overnight at 4˚C. The membrane was then washed with PBS and incubated with horseradish peroxidase-conjugated immunoglobulin G secondary antibody (1:10,000) for 1 h at 37˚C. Blots were developed using a SuperSignal chemiluminescence detection kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and immunoblotting was visualized and analyzed using the Quantity One System 4.62 (Bio-Rad, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. Differences among groups were evaluated by one-way analysis of variance followed by a Dunnett's t-test for multiple comparisons. $P<0.05$ was determined to indicate a statistically significant difference.

Results

PTB decreases myocardial infarct size via PI3K/Akt signaling pathway activation. A myocardial infarct was induced all groups apart from the Sham group (Fig. 1). Treatment with PTB significantly decreased the myocardial infarct size compared

with the M/IR group (P<0.05; Fig. 1). However, the protective effect of PTB was reversed by LY treatment, as the myocardial infarct size was significantly increased in the MI/R+PTB+LY group compared with the MI/R+PTB group (P<0.05; Fig. 1).

PTB decreases the release of myocardial CK-MB and LDH following MI/R via PI3K/Akt signaling pathway activation. The release of CK-MB and LDH is an indicator of myocardial injury; therefore, serum levels of CK-MB and LDH were measured. There was a significant increase in the release of serum CK-MB and LDH in the MI/R group compared with the Sham group (both P<0.05; Fig. 2). However, there was a significant decrease in the release of CK-MB and LDH in the MI/R+PTB group compared with the MI/R group (both P<0.05; Fig. 2). The release of CK-MB and LDH was significantly increased in the MI/R+PTB+LY group compared with the MI/R+PTB group (P<0.05; Fig. 2) indicating that PI3K was involved in the protective effect of PTB against MI/R injury.

PTB decreases myocardial MPO activity and the serum levels of TNF-α, IL-6 and IL-8 following MI/R via activation of the PI3K/Akt signaling pathway. Increased MPO activity in the myocardium indicates neutrophil infiltration. MPO activity in the MI/R group was significantly increased compared with the Sham group (P<0.05; Fig. 3A). However, PTB significantly decreased myocardial MPO activity in the MI/R+PTB group compared with the MI/R group and LY treatment reversed the effect of PTB (both P<0.05; Fig. 3A). Levels of TNF-α, IL-6, and IL-8 in the plasma were all significantly increased following MI/R injury compared with the Sham group (all P<0.05; Fig. 3B-D). Treatment with PTB significantly decreased cytokine levels in the serum (all P<0.05; Fig. 3B-D) and these effects were reversed following LY treatment (all P<0.05; Fig. 3B-D).

PTB reduces myocardial apoptosis induced by MI/R via activation of the PI3K/Akt signaling pathway. TUNEL staining was performed to evaluate apoptosis in the rat hearts (Fig. 4). The apoptotic index of the MI/R+PTB group was significantly decreased compared with the MI/R group (P<0.05; Fig. 4). However, treatment with LY again significantly reversed the protective effects of PTB (P<0.05; Fig. 4), indicating that PI3K activation is involved in the mechanism of action of PTB against MI/R-induced myocardial apoptosis.

Effects of PTB on the expression of p-Akt, Akt, Bcl-2 and Bax in hearts subjected to MI/R. MI/R injury in the MI/R group significantly increased the phosphorylation of Akt compared with the Sham group (P<0.05; Fig. 5A and B). The phosphorylation of Akt was further increased following treatment with PTB (P<0.05, Fig. 5A and B). However, treatment with LY reversed the increase in Akt phosphorylation that was induced by PTB (P<0.05; Fig. 5A and B). In addition, MI/R injury significantly decreased Bcl-2 expression in the MI/R group compared with the Sham group (P<0.05; Fig. 5C). Treatment with PTB significantly increased the expression of Bcl-2 (P<0.05), which was subsequently decreased by co-administration with LY (both P<0.05; Fig. 5C). By contrast, MI/R injury induced a significant increase in Bax expression compared with the Sham group (P<0.05; Fig. 5D). PTB administration significantly decreased the expression of Bax in the MI/R group compared with the MI/R+PTB group (P<0.05), which was significantly reversed by co-administration with LY (both P<0.05; Fig. 5D).

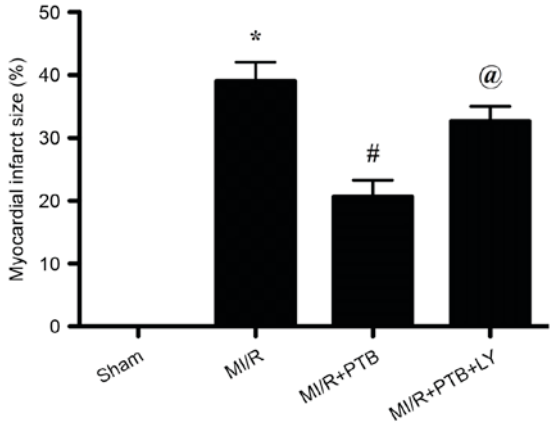

Figure 1. PTB treatment decreased myocardial infarct size via the phosphatidylinositol 3'-kinase-protein kinase B signaling pathway. Data are expressed as the mean ± standard error of the mean (n=6 for each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the MI/R group and @P<0.05 vs. the MI/R+PTB group. PTB, pterostilbene; MI/R, myocardial ischemia/reperfusion; LY, LY294002.

Discussion

The current study demonstrated that myocardial I/R injury is attenuated by treatment with PTB. PTB decreased cardiac injury, alleviated myocardial apoptosis and decreased the inflammation induced by myocardial I/R injury. The protective effect of PTB is strongly associated with activation of the PI3K/Akt signaling pathway. Treatment with PTB significantly decreased the release of CK-MB and LDH and the activity of MPO following myocardial I/R injury. PTB administration also decreased levels of TNF-α, IL-6, and IL-8 in the serum. Furthermore, treatment with PTB reduced myocardial apoptosis. However, the cardioprotective effects of PTB were reversed following treatment with the PI3K signaling inhibitor LY, suggesting that activation of the PI3K/Akt signaling is involved in the cardioprotective effects of PTB.

CK is an enzyme expressed in various types of tissues and its isoenzyme is CK-MB in the myocardium. LDH is an enzyme that catalyzes the conversion of lactate to pyruvic acid. The isoenzyme of LDH in the heart is LDH-1 and may be used to identify damage in the heart (13). Following cardiomyocyte damage, cellular CK-MB and LDH are released into the blood, resulting in increased levels of CK-MB and LDH in the serum. The results of the present study demonstrate that the serum levels of CK-MB and LDH are decreased following PTB treatment, suggesting that PTB treatment alleviates myocardial injury following I/R.

MPO is a peroxidase enzyme, which is most abundantly expressed in neutrophils. Therefore, MPO activity in the myocardium is regarded as an indicator of neutrophil infiltration and inflammation. The current study demonstrated that PTB treatment decreased MPO activity in the myocardium, suggesting that PTB may attenuate neutrophil

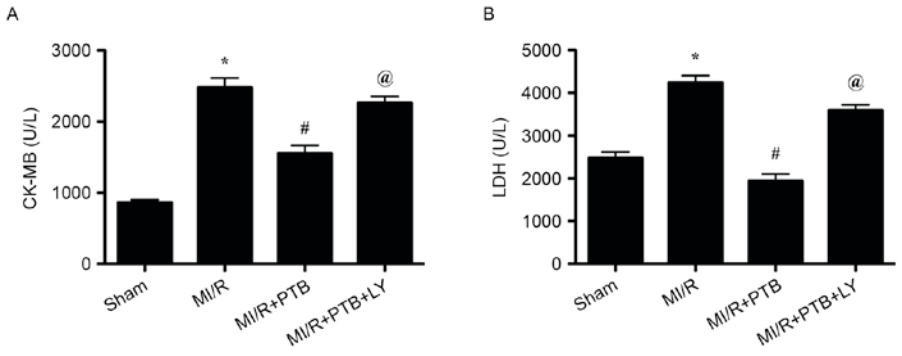

Figure 2. PTB treatment reduced levels of CK-MB and LDH in the serum via the phosphatidylinositol 3'-kinase-protein kinase B signaling pathway. (A) Serum CK-MB. (B) Serum LDH. Data are expressed as the mean ± standard error of the mean (n=6 for each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the MI/R group and @P<0.05 vs. the MI/R+PTB group. PTB, pterostilbene; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; MI/R, myocardial ischemia/reperfusion; LY, LY294002.

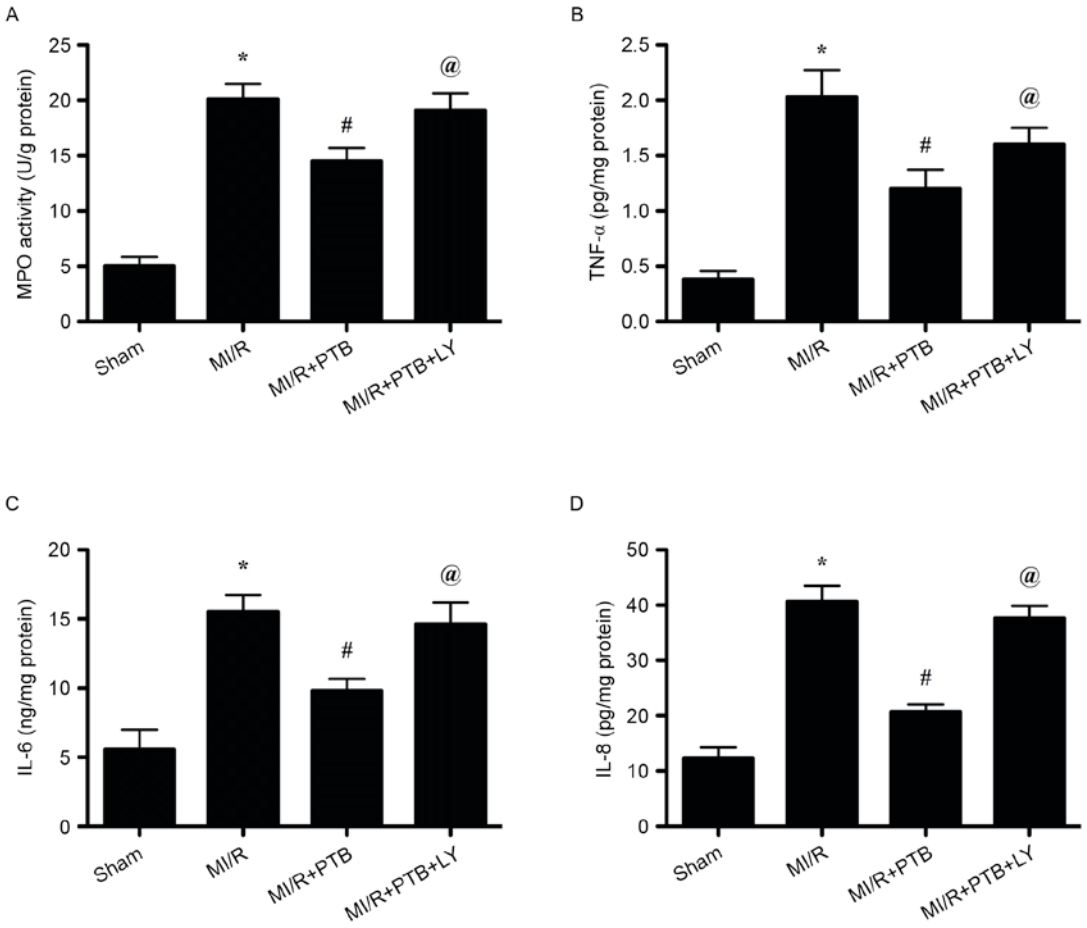

Figure 3. PTB treatment reduced levels of inflammatory cytokines via the phosphatidylinositol 3'-kinase-protein kinase B signaling pathway. (A) Myocardial MPO activity. (B) Serum TNF-α. (C) Serum IL-6. (D) Serum IL-8. Data are expressed as the mean ± standard error of the mean (n=6 for each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the MI/R group and @P<0.05 vs. the MI/R+PTB group. PTB, pterostilbene; MPO, myocardial myeloperoxidase; TNF-α, tumor necrosis factor α; IL, interleukin; MI/R, myocardial ischemia/reperfusion; LY, LY294002.

infiltration and the inflammatory response following MI/R injury. Inflammation serves an important role in MI/R injury. Damaged cells stimulate inflammatory cells, which subsequently release various inflammatory cytokines. The release of cytokines causes further injury to endothelial cells, resulting in increased vascular permeability (14). Therefore, in the current study, serum levels of TNF-α, IL-6, and IL-8 were measured to determine the anti-inflammatory effect of PTB. The results demonstrated that PTB treatment deceases serum levels of TNF-α, IL-6, and IL-8. Overall, these results demonstrate that PTB decreases neutrophil infiltration and the inflammatory response during myocardial I/R injury.

Myocardial apoptosis also serves an important role in myocardial I/R injury. It is inhibited by Bcl-2 and induced by Bax (15). The effect of PTB on myocardial apoptosis was assessed using TUNEL staining in the current study. The

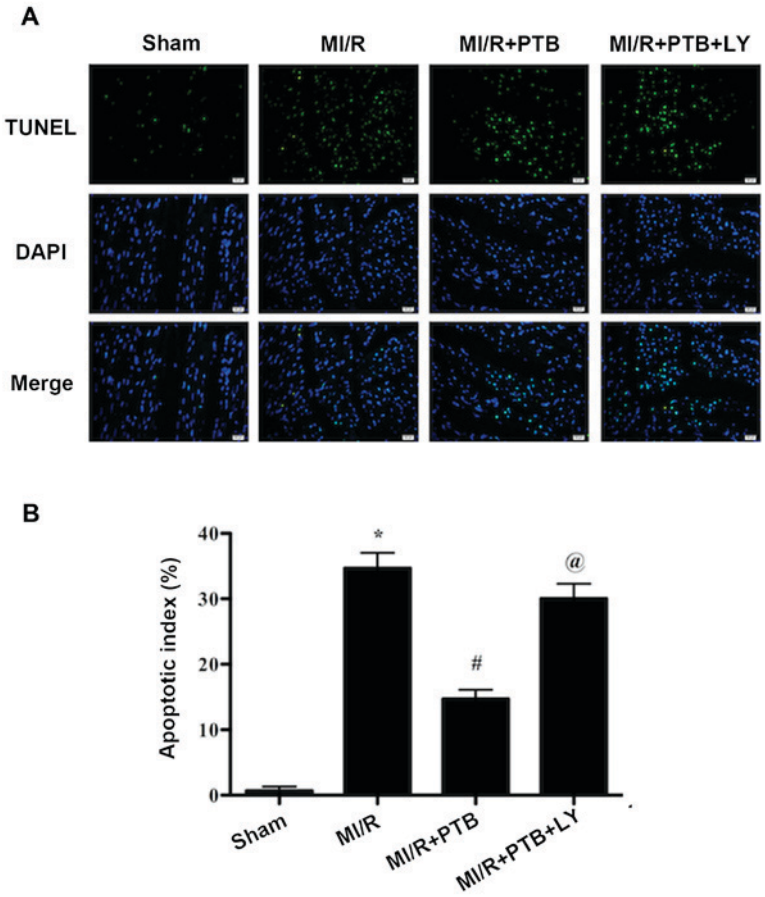

Figure 4. PTB treatment decreased myocardial apoptosis via phosphatidylinositol 3'-kinase-protein kinase B signaling. (A) TUNEL and DAPI staining of myocardial tissues from all groups. Magnification, x400. (B) Apoptosis index. Data are expressed as the mean ± standard error of the mean (n=6 for each group). *P<0.05 vs. the Sham group, #P<0.05 vs. the MI/R group and @P<0.05 vs. the MI/R + PTB group. PTB, pterostilbene; MI/R, myocardial ischemia/reperfusion; LY, LY294002; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

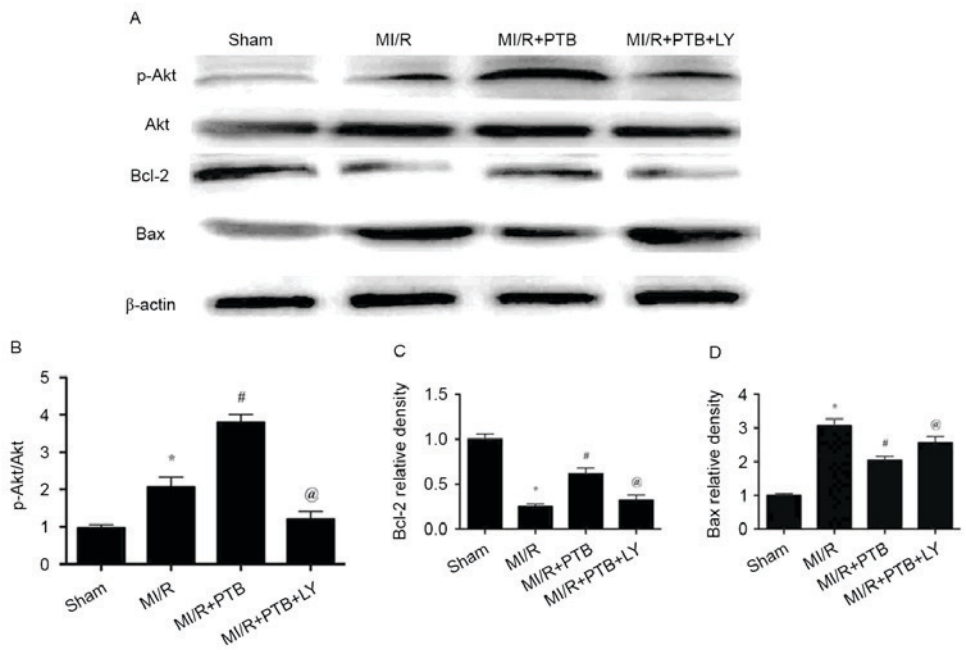

Figure 5. PTB treatment activated PI3K/Akt signaling and inhibited myocardial apoptosis signaling, which was reversed by LY treatment. (A) Western blot analysis measuring the expression of p-Akt, Akt, Bcl-2, Bax and β-actin in all groups. (B) Expression of P-Akt/Akt. (C) Expression of Bcl-2. (D) Expression of Bax. Data are expressed as the mean ± standard error of the mean (n=6 for each group). *P<0.05 vs. the Sham group; #P<0.05 vs. the MI/R group and @P<0.05 vs. the MI/R + PTB group. PTB, pterostilbene; MI/R, myocardial ischemia/reperfusion; LY, LY294002; PI3K/Akt, phosphatidylinositol 3'-kinase-protein kinase B; Akt, protein kinase B; p, phosphorylated.

results indicate that PTB treatment reduces the number of TUNEL-positive cells. Furthermore, levels of Bcl-2 and Bax were measured using western blotting and the results demonstrated that PTB treatment increases Bcl-2 expression and decreases Bax expression. However, the protective effects of PTB were inhibited by LY, a PI3K signaling inhibitor.

PI3K/Akt signaling is strongly associated with cell survival (16,17). Previous studies have demonstrated that PI3K/Akt signaling confers significant cardioprotective effects (18,19). Akt phosphorylation suppresses apoptosis and promotes cell survival in myocardial ischemia (20-22). The results of the current study demonstrate that PTB treatment upregulates Akt phosphorylation, indicating that the protective effects of PTB are associated with activation of the PI3K/Akt signaling pathway. Treatment with LY reverses the increase in Akt phosphorylation, further confirming this conclusion.

In conclusion, the present study demonstrates that PTB, an antioxidant and anti-inflammatory agent, attenuates myocardial I/R injury. The results also indicate that the activation of the PI3K/Akt signaling pathway by PTB protects against myocardial I/R injury. Thus, these findings may facilitate the development of PTB as a therapeutic strategy to treat myocardial I/R injury.

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