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EXPERIMENTAL STUDY

Effect of aqueous extract of Sanweitanxiang powder on calcium homeostasis protein expression in ischemic-reperfusion injury rat heart

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Abstract

OBJECTIVE: To investigate the underlying mechanism of reduced myocardial ischemia-reperfusion (I/R) injury in rats using the traditional Tibetan medicine Sanweitanxiang powder (SWTX).

METHODS: Rats were randomly divided into six groups (n=10) as follows: (a) propranolol dinitrate control group, given propranolol dinitrate 0.02 g/kg for 10 days before I/R, (b) SWTX with a high dose group, given SWTX 1.5 g/kg for 10 days before I/R, (c) SWTX with a medium dose group, given SWTX 1.25 g/kg for 10 days before I/R, (d) sham group (Sham), in which the rat heart was exposed by pericardiotomy but without I/R, (e) SWTX with a low dose group, given SWTX 1.0 g/kg for 10 days before I/R, and (f) I/R injury group. Rats were intragastrically pretreated with propranolol dinitrate or SWTX. After that, the operation to cause ischemia and reperfusion was conducted. The histopathologic changes of rat hearts were observed by hematoxylin and eosin staining and transmission electron microscopy. Ca^{2+} homeostasis protein expression was determined by western blot.

RESULTS: After SWTX pretreatment, the development of ultrastructural pathological changes from IR injury was attenuated. A decrease in the expression of B-cell lymphoma 2 associated X protein, and an increase in the expression of B-cell lymphoma 2 were observed. An increased activation of extracellular signal regulated kinases were found. Compared with the sham group, the expression of sarcoplasmic reticulum calcium-ATPase, phospholamban, and calsequestrin were all up-regulated after pretreatment with SWTX.

CONCLUSION: The protective mechanism of SWTX pretreatment on myocardial I/R injury might be related to its effect on maintaining the balance of calcium homeostasis in rat heart.

Key words: Reperfusion injury; Homeostasis; Protein expression; Sanweitanxiang powder

INTRODUCTION

Myocardial ischemia injury is a major consequence of cardiovascular disease. Although the pathogenesis of myocardial ischemic injury was reported in 1960, unresolved issues still exist.[1-3] Percutaneous coronary intervention (PCI) is one of the most effective methods

when coronary occlusion is relieved within a critical time window.[4-6] However, reperfusion has profound and dangerous effects on ischemic myocardium, which are referred to as myocardial ischemia-reperfusion (I/R) injury.[7-9] Many therapeutic approaches have been explored to protect against myocardial I/R injury, such as preconditioning and postconditioning.[10-14] Among them, pharmacological preconditioning which attempts to attenuate I/R injury can be used as an effective adjunct to PCI and reperfusion.[15-17]

A number of Traditional Tibetan Medicines have been used in the treatment of cardiovascular diseases with positive effects, providing a basis for exploring pharmacological candidates for the treatment of I/R injury.[18] Sanweitanxiang powder (SWTX) consists of Tanxiang (*Lignum Santali Albi*), Guangzao (*Fructus Choerospondiatis*), and Roudoukou (*Semen Myristicae Fragrantis*). SWTX has been widely used in ethnomedicine for clinical therapy of cardiopyretic disease and is used by the Qinghai and Tibetan traditional healers.[19]

In our previous research,[19] we found that pretreatment with SWTX reduced I/R injury in rat hearts. We observed that the myocardial infarction area decreased. The levels of malondialdehyde (MAD), nitric oxide synthase (NOS), inducible NOS, and NO were down-regulated and superoxide dismutase, and glutathione peroxidase were up-regulated in serum. The myocardial contraction and relaxation ability was enhanced after SWTX pretreatment. However, the mechanisms of SWTX pretreatment remain elusive. We hypothesized that SWTX pretreatment induced cardioprotection by maintaining Ca^{2+} homeostasis during I/R injury. Therefore, we assayed the influence of SWTX on the expression of proteins involved in Ca^{2+} homeostasis.

MATERIALS AND METHODS

Medicines and reagents

SWTX was purchased from the Tibetan Medicine Corporation (Qinghai, China). A known quantity of dried SWTX was soaked in distilled water for 24 h at 35℃-42℃ and macerated with a mortar and pestle. The mixture was filtered, condensed with a rotary evaporator, and lyophilized. The dry powder was dissolved in distilled water at concentrations of 15% , 12.5% , and 10% for use. Propranolol dinitrate was purchased from Shanxi SANPU Pharmaceutical Corporation (Shanxi, China).

The antibodies for extracellular signal regulated kinase (ERK) (sc-94), phosphorylation of ERK (sc-81492), B-cell lymphoma 2 (Bcl-2) (sc-7382), sarcoplasmic reticulum calcium-ATPase (SERCA2a) (sc-8094), and phospholamban (sc-17024-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies for calsequestrin (ab3516) and Bcl-2 associated X protein (Bax) (ab7977) were purchased from Abcam Corporation (Cambridge, MA, USA). ECL chemiluminescence kit was purchased from Biyuntian Biotech Institute (Beijing, China).

Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Experimental Animal Center at the medical college of Lanzhou, China (Certificate of quality: SCXK, 2009-0004). The animals were housed with a 12 h light-dark cycle at 25℃± 2℃, and in a relative humidity of 30%-60%. Rats were fed *ad libitum* on a diet of standard pellets and water. Experimental rats were randomly divided into six groups (n=10): (a) propranolol dinitrate control group (PC), given propranolol dinitrate 0.02 g/kg for 10 days before I/R, (b) SWTX with a high dose group (HS), given SWTX 1.5 g/kg for 10 days before I/R, (c) SWTX with a medium dose group (MS), given SWTX 1.25 g/kg for 10 days before I/R, (d) sham group (Sham), in which rat hearts were exposed by pericardiotomy but without I/R, (e) SWTX with a low dose group (LS), given SWTX 1.0 g/kg for 10 days before I/R, and (f) I/R injury group. Rats were pretreated intragastrically with Propranolol dinitrate or SWTX.

In vivo experimental protocol

All animals were anesthetized with a single intraperitoneal injection of urethane (1.0 g/kg). After tracheal intubation, rats were ventilated by an animal artificial respirator (DH1, Medical Equipment Produce Company in Medical College of Zhejiang, China). The breathing frequency was maintained at 55-65 breaths/min. A lead-II electrocardiogram was used via subcutaneous stainless steel electrodes (American Biopac System). The body temperature of rats was maintained at 38℃ during the entire experiment process.

Ischemia and reperfusion induction

After performing a left thoracotomy and pericardiotomy, the hearts were exposed. A suture was passed around the left anterior descending coronary artery by inserting a small curved prolene 6.0 needle into the margin of the pulmonary cone, exiting through the middle of the line linking the cone to the atrium. The suture ends were threaded through a small vinyl tube to prepare a snare. After surgical preparation, the rat was allowed to stabilize for 20 min. In the experimental groups, the left anterior descending coronary artery was occluded for 30 min by tightening the snare. Myocardial ischemia was confirmed by the appearance of a regional cyanosis and akinesia or bulging on the epicardium distal to the snare. After 30 min of ischemia, the snare was released and reperfusion was allowed for a period of 40 min. Hemodynamic parameters were measured at: (a) 0 min before ischemia (baseline), (b) at the end of ischemia (or no ischemia), and (c) at the end of 40 min reperfusion.

Hematoxylin and eosin (HE) staining

After 40 min reperfusion, the heart tissues were fixed in 10% formalin for at least 24 h, embedded in paraffin, and cut into 5 μm-thick sections using a rotary mi-

crotome. The sections were stained with HE dye and observed under a microscope to detect histopathological changes in the heart tissue.

Transmission electron microscopy

Small heart tissue samples about 1 mm^3 were fixed in 4% glutaraldehyde, postfixed for 1 h in 1% OsO_4 in 0.1 M cacodylate buffer, dehydrated, and embedded in Epon 812 at 60℃ for 48 h. Routine 60 nm ultrathin sections were cut and mounted on coated grids, and stained with 1% uranyl acetate and Reynold's lead citrate. Transmission electron microscopy was performed with H 600 at 60 kV.

Western blot analysis

After the I/R experiment, the heart tissue was obtained and total protein was extracted from the risk zones. The myocardial tissue was placed in lysis buffer containing protease inhibitors, homogenized, and centrifuged. The protein concentration of each sample was determined using a bicinchoninic acid protein assay kit. To detect the expression of ERK, phosphorylation of ERK, Bcl-2, Bax, SERCA2a, phospholamban (PLB), and calsequestrin (CQS), 20 μg of the total protein were electrophoresed on a 4%-12% polyacrylamide gel. The gels were transferred to polyvinylidene difluoride (PVDF) membranes by electrophoretic transfer and blocked for 1 h in 5% skim milk. The samples were incubated with primary antibodies (1∶200) in Tris Buffered Saline and Tween-20 (TBST) with 5% bovine serum albumin (BSA) overnight at 4℃. They were then incubated with IgG-horseradish peroxidase-conjugated secondary antibody (1∶5000) in TBST with 1% BSA. The signals were detected by ECL.

Statistical analysis

All data were expressed as the mean±*SEM* of results obtained from the experiments. Differences between data sets were assessed by one-way analysis of variance followed by Newman-Keuls tests (SPSS 19, Armonk, NY, USA). $P<0.05$ was considered as statistically significant.

RESULTS

SWTX pretreatment on I/R injury heart histomorphology

Sections from the sham group displayed normal structure without pathologic changes seen under a light microscope. There was no leukocyte infiltration or necrosis in the sham group (Figure 1A). In the I/R injury group, marked histopathologic changes were observed, such as coagulation necrosis, karyolysis, disappearance of the myocardial tissues, and apparent inflammatory cell infiltration (Figure 1B). The extent of inflammatory lesions in the LS group was significantly less severe than in the I/R injury group (Figure 1C). Myocardial tissue samples observed with transmission electron microscopy are shown in Figure 1D-F. Samples exhibited a normal morphologic appearance in the sham group. The myocytes have well-ordered myofibrils with a distinct sarcomeric registry and dark mitochondria with tightly arranged cristae (Figure 1D). In contrast, hearts subjected to the I/R treatment showed abnormalities in mitochondrial and myofilament morphology. The 40 min I/R exhibited evidence of severe, irreversible injury, including breaks in the sarcolemma, abnormal nuclei, hyperplasia of mitochondria, and dense intramitochondrial granules. Swelling and paucity of cristae with loss of cytosolic glycogen were also found in mitochondria (Figure 1E). In the LS group, the myocytes showed well-ordered myofibrils and the mitochondria showed well-ordered arranged crista, although with a slight paucity (Figure 1F).

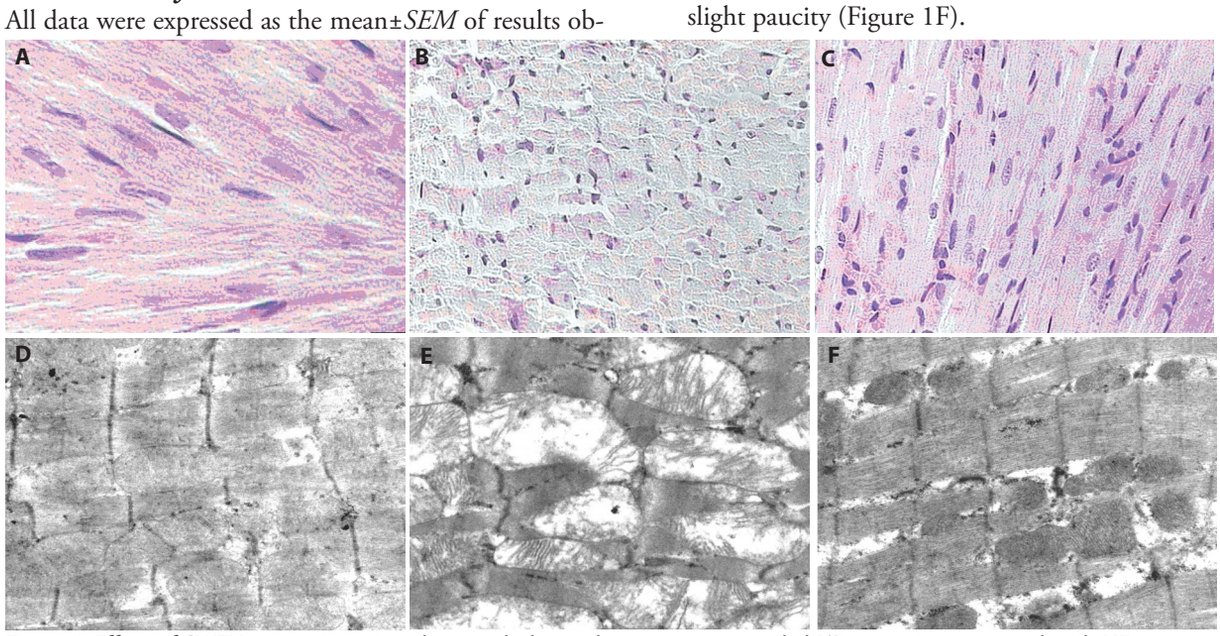

Figure 1 Effect of SWTX pretreatment on histopathologic changes in myocardial I/R injury in rats tested with HE staining (A-C, ×200) and transmission electron microscopy (D-F, ×10 000)

A, D: sham group; B, E: I/R injury group; C, F: LS group, given SWTX 1.0 g/kg for 10 days before I/R. I/R: ischemia-reperfusion; LS: SWTX with a low dose; SWTX: Sanweitanxiang powder; HE: hematoxylin and eosin.

SWTX pretreatment on activity of proteins related to ERK

The phosphorylation activity of ERK was higher in I/R injured hearts than in the sham group. Compared with p44 ERK, p42 ERK was not activated significantly in the I/R injury group. However, rat myocardium pretreated with 1.5 g/kg SWTX showed dramatically higher levels of phosphorylation activity of ERK when compared with I/R injury group and PC group (Figure 2).

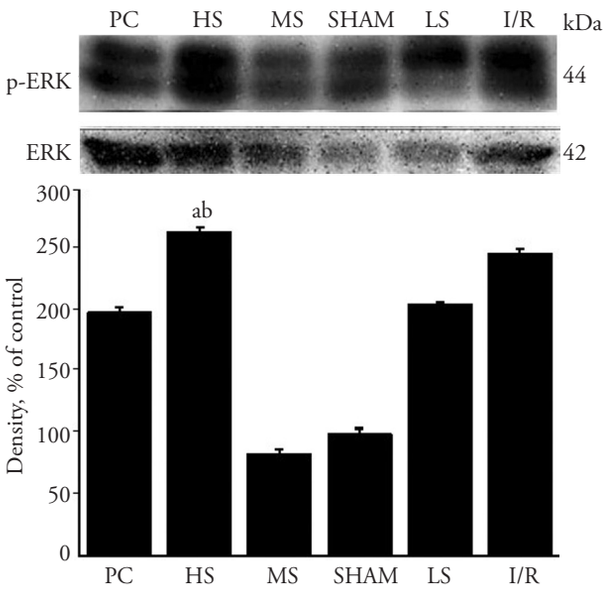

Figure 2 Western blotting of SWTX pretreatment on phosphorylation activity of ERK

PC: propranolol dinitrate control group, given propranolol dinitrate 0.02 g/kg for 10 days before I/R; HS: SWTX with a high dose group, given SWTX 1.5 g/kg for 10 days before I/R; MS: SWTX with a medium dose group, given SWTX 1.25 g/kg for 10 days before I/R; Sham: sham group, rats heart was exposed by pericardiotomy but without I/R; LS: SWTX with a low dose group, given SWTX 1.0 g/kg for 10 days before I/R; I/R: I/R injury group. SWTX: Sanweitanxiang powder; ERK: extracellular signal-regulated kinases; I/R: ischemia-reperfusion. Each signal was quantified by scanning densitometry and data denote the means±*SEM* of 2-3 replicate measurements in six different groups. Compared with the sham group, $^{a}P<0.01$; compared with I/R injury group, $^{b}P<0.01$.

SWTX pretreatment on expression of proteins related to apoptosis

As shown in Figure 3, I/R reduced Bcl-2 expression and increased Bax expression. In the SWTX-pretreated groups, the expression of Bax decreased and Bcl-2 increased compared with the sham group ($P<0.05$).

SWTX pretreatment on expression of proteins related to calcium homeostasis

As shown in Figure 4, I/R induced a marked decrease in SERCA2a protein levels compared with the sham group. In the SWTX pretreated group, this decrease of SERCA2a was attenuated by pharmaceutical preconditioning, but was still lower than that in the sham group. PLB expression was increased significantly after SWTX pretreatment as compared with the sham and I/R groups ($P<0.05$). CQS expression increased after SWTX pretreatment, but was still lower than that in the I/R group.

DISCUSSION

ERK 1/2 are members of the mitogen-activated protein kinases, a family of serine-threonine kinases that regulate cell proliferation, differentiation, and survival.[20] ERK 1/2 are activated in response to I/R, oxidative stress, and hypoxia,[21] And ERK 1/2 have been shown to mediate cellular protection during I/R.[22] We measured the phosphorylation of ERK (42 and 44 kDa) by western blot. Our results indicate that SWTX pretreatment activated ERK 1/2 which could maintain myocardial viability in hearts exposed to I/R injury.

Apoptosis is one of the main causes of damage after myocardial I/R injury. It has been reported that the mitochondrial permeability transition pore opening causes heart damage by apoptosis.[23] The anti-apoptotic protein Bcl-2 has been known to attenuate cellular injury by preventing injurious Ca^{2+} release from the endoplasmic reticulum and inhibiting Bax translocation from the cytoplasm to mitochondria.[24] Activation of ERK 1/2 also inhibits the conformational change in Bax protein thereby preventing apoptosis.[25] In our study, we found an increase in expression of Bcl-2 and a decrease in Bax after pretreatment with SWTX which could protect against apoptosis induced by I/R injury and therefore prevent the heart from injury by reperfusion.

Ca^{2+} is an important messenger in intracellular signal transduction and plays an essential role in cardiac excitation-contraction coupling. During the process, intracellular Ca^{2+} homeostasis is carefully regulated by ion channels, specific binding, and transport proteins. Intracellular dysregulation of Ca^{2+} homeostasis is proposed as the mechanism of cell injury induced by I/R.[26] The interruption of Ca^{2+} overload has been proposed as an important target for increasing tolerance to I/R injury.[27] Important Ca^{2+} cycling proteins such as SERCA2a, PLB, and CQS in the sarcoplasmic reticulum play a central role in regulating the intracellular Ca^{2+} concentration.[28] SERCA2a is the most abundant Ca^{2+}-ATPase within the myocardium and the PLB is one of the key SERCA2a regulatory proteins.[29] Our previous studies have demonstrated that SWTX pretreatment contributes to protection against I/R injury, but the effects on Ca^{2+} cycling proteins have not been investigated.[19] In this study, we found that I/R injury markedly decreases SERCA2a protein expression, and pretreatment with SWTX could increase the expression. Meanwhile, pretreatment with SWTX at a dose of 1.5 g/kg significantly increased PLB expression. The protective mechanism of SWTX pretreatment could be related to Ca^{2+} homeostasis by increasing protein content of Ca^{2+}-cycling proteins in the sarcoplasmic reticulum. Some re-

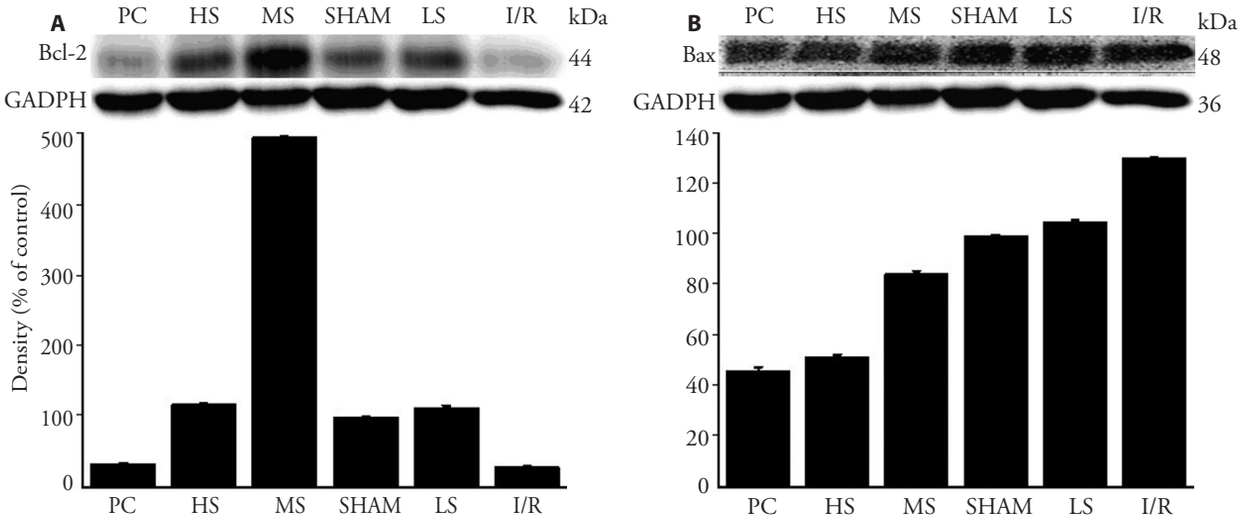

Figure 3 Western blotting of SWTX pretreatment on the expression of Bcl-2 (A) and Bax proteins (B)
PC: propranolol dinitrate control group, given propranolol dinitrate 0.02 g/kg for 10 days before I/R; HS: SWTX with a high dose group, given SWTX 1.5 g/kg for 10 days before I/R; MS: SWTX with a medium dose group, given SWTX 1.25 g/kg for 10 days before I/R; Sham: sham group, rats heart was exposed by pericardiotomy but without I/R; LS: SWTX with a low dose group, given SWTX 1.0 g/kg for 10 days before I/R; I/R: I/R injury group. SWTX: Sanweitanxiang powder; Bcl-2: B-cell lymphoma 2; Bax: Bcl-2 associated X protein; GAPDH: glyceraldehyde phosphate dehydrogenase; I/R: ischemia-reperfusion. Each signal was quantified by scanning densitometry. Data denote the mean±*SEM* of 2-3 replicate measurements in six different groups, $P<0.05$.

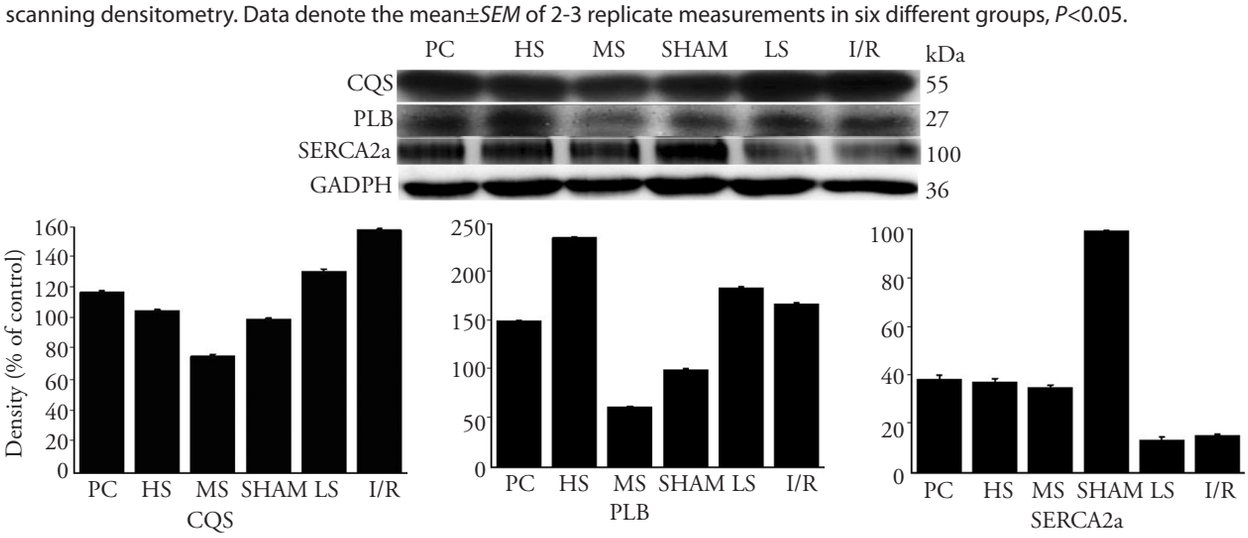

Figure 4 Western blotting of SWTX pretreatment on the expression of CQS, PLB, and SERCA2a proteins
PC: propranolol dinitrate control group, given propranolol dinitrate 0.02 g/kg for 10 days before I/R; HS: SWTX with a high dose group, given SWTX 1.5 g/kg for 10 days before I/R; MS: SWTX with a medium dose group, given SWTX 1.25 g/kg for 10 days before I/R; Sham: sham group, rats heart was exposed by pericardiotomy but without I/R; LS: SWTX with a low dose group, given SWTX 1.0 g/kg for 10 days before I/R; I/R: I/R injury group. SWTX: Sanweitanxiang powder; CQS: Calsequestrin, PLB: Phospholamban; SERCA2a: sarcoplasmic reticulum Ca^{2+}-ATPase; GAPDH: glyceraldehyde phosphate dehydrogenase. Each signal was quantified by scanning densitometry. Data denote the mean±*SEM* of 2-3 replicate measurements in six different groups. $P<0.05$.

search has also suggested that changes in Ca^{2+} homeostasis play an important role in the modulation of apoptosis.[30] The increased expression of Ca^{2+} homeostasis proteins could also protect myocardial cells from apoptosis.

In summary, our study demonstrated that SWTX pretreatment protects against I/R injury in rat hearts by activating ERK 1/2 and inhibiting apoptosis. Furthermore, our results showed that the protective activities of SWTX pretreatment might be related to the expression of proteins involved in Ca^{2+} homeostasis. Further research on elucidating the mechanism in detail is ongoing.

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