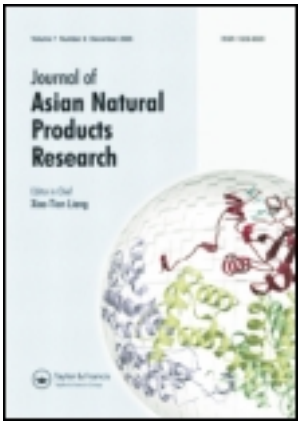

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

Tribulosin suppresses apoptosis via PKC epsilon and ERK1/2 signaling pathway during hypoxia/reoxygenation in neonatal rat ventricular cardiac myocytes

Shuang Zhang [a], Hong Li [a] & Shi-Jie Yang [a]

[a] Department of Pharmacology, Norman Bethune College of Medicine, Jilin University, Changchun, 130021, China

Available online: 25 Nov 2011

To cite this article: Shuang Zhang, Hong Li & Shi-Jie Yang (2011): Tribulosin suppresses apoptosis via PKC epsilon and ERK1/2 signaling pathway during hypoxia/reoxygenation in neonatal rat ventricular cardiac myocytes, Journal of Asian Natural Products Research, 13:12, 1135-1145

To link to this article: http://dx.doi.org/10.1080/10286020.2011.627327

Journal of Asian Natural Products Research
Vol. 13, No. 12, December 2011, 1135–1145

Tribulosin suppresses apoptosis via PKC epsilon and ERK1/2 signaling pathway during hypoxia/reoxygenation in neonatal rat ventricular cardiac myocytes

Shuang Zhang, Hong Li and Shi-Jie Yang*

Department of Pharmacology, Norman Bethune College of Medicine, Jilin University, Changchun 130021, China

(*Received 14 April 2011; final version received 23 September 2011*)

Tribulosin (tigogenin 3-*O*-β-D-xylopyranosyl(1–2)-[β-D-xylopyranosyl (1–3)]-β-D-glucopyranosyl (1–4)-[a-L-rhamnopyranosyl(1–2)]-β-D-galactopyranoside), a component of *gross saponins of Tribulus terrestris*, has been shown to produce cytoprotective effects in heart. Yet, the precise mechanisms are not fully understood. We examined the mechanisms of tribulosin on myocardial protection. Ventricular myocytes were isolated from the heart of neonatal rats and were exposed to 3 h of hypoxia followed by 2 h reoxygenation. Apoptosis was induced by hypoxia/reoxygenation (H/R), and the expression of protein kinase C epsilon (PKCε) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) in cultured neonatal rat cardiac myocytes was detected. The results indicated that treatment with tribulosin in the culture medium protected cardiac myocytes against apoptosis induced by H/R. PKCε and ERK1/2 expression increased after pretreated with tribulosin. In the presence of PKCε inhibitor co-treated with tribulosin, the expression of ERK1/2 was decreased in H/R cardiac myocytes. While preconditioned with PD98059, ERK1/2 inhibitor, no effects on the expression of PKCε were detected. Tribulosin has protective effects on cardiac myocytes against apoptosis induced by H/R injury via PKCε and ERK1/2 signaling pathway.

Keywords: tribulosin; myocytes; hypoxia/reoxygenation injury; apoptosis; PKC epsilon; ERK1/2

1. Introduction

Tribulus terrestris is a traditional Chinese plant which has many activities. Gross saponins of *Tribulus terrestris* (GSTT) comprised of spiral vagina sterol and snail sterol are well-known Chinese medicine used for the treatment of various diseases including coronary heart disease, hypertension, hyperlipidemia, and platelet aggregation. The mechanisms by which GSTT protects the ischemic heart have been extensively investigated in our laboratory for many years [1–5]. However, up to now, little is known about which specific component in GSTT plays the important role. We have identified nine saponin monomers and named them A, B, C, D, E, F, G, J, and I transitorily. Each of them is a kind of spiral vagina steroid. After preliminary screening, we determined that saponin monomer B is bioactive [6,7]. *T. terrestris* saponin B is a spiral vagina sterol whose chemical name is tigogenin 3-*O*-β-D-xylopyranosyl(1–2)-[β-D-xylopyranosyl (1–3)]-β-D-glucopyranosyl(1–4)-[a-L-rhamnopyranosyl(1–2)]-β-D-galactopyranoside (Figure 1). It is also called tribulosin, which has been shown to produce cytoprotective effects on cardio-

*Corresponding author. Email: jcyaoli@sina.com

ISSN 1028-6020 print/ISSN 1477-2213 online

http://dx.doi.org/10.1080/10286020.2011.627327
http://www.tandfonline.com

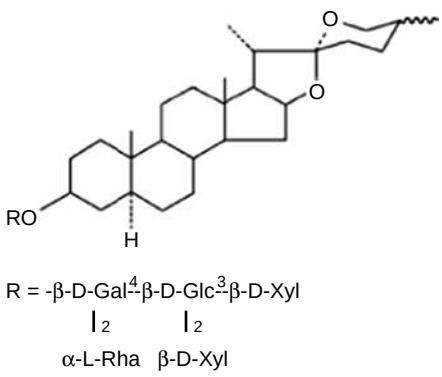

Figure 1. Structure of tribulosin.

myocytes [6–8]. Yet, the precise mechanisms are not fully understood. Here, we looked for the mechanisms for the protective effect of tribulosin on cardiomyocytes against hypoxia/reoxygenation (H/R) injury in cultured neonatal rat cardiomyocytes.

Our previous study [8] has shown that tribulosin attenuates myocardial ischemia/reperfusion (I/R) injury and inhibits cardiac myocyte apoptosis related to protein kinase C epsilon (PKCε). Although PKCε appears to play an essential role in the protective effect of tribulosin, the downstream signaling events of this specific isozyme remain largely unknown. A plausible target for PKC-mediated signaling events is the family of mitogen-activated protein kinases (MAPKs) [9]. Evidence indicates that extracellular signal-regulated kinase (ERK) signaling is required to protect the myocardium from I/R injury *in vivo* [9,10]. In cardiac tissue, activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) has been shown to be a necessary event during ischemic, and activation of ERK1/2 has been observed after brief ischemia.

2. Results and discussion

2.1 Biochemical parameters

In this study, we mimicked the I/R-injury process *in vivo*, and assayed some indices of cardiac myocyte injury and elimination interference from body fluid, and thus investigated the effects of tribulosin on H/R-induced apoptosis and explored the cardioprotective mechanisms of tribulosin. The results demonstrated that aspartate aminotransferase (AST), malondialdehyde (MDA), and the rate of cardiac myocyte apoptosis in the H/R group were higher than those in the control group, which indicated that the H/R model was made successfully *in vitro*. However, through the procedure of pre-treatment of tribulosin, the above-mentioned indices were obviously lower than those in the H/R group, but higher than those in the control group. After pre-treatment with PKCε inhibitor and ERK1/2 inhibitor, the inhibitory action was suppressed (Table 1).

The results demonstrated that tribulosin relieved injury induced by the H/R process significantly and improved cell viability through the suppression of apoptosis, thereby providing protection to cardiac myocytes.

2.2 Survival rate

The survival rate of cardiac myocytes decreased after H/R injury. When pretreated

Table 1. Effect of tribulosin on AST and MDA in the culture medium after cardiac cells impaired by H/R ($n = 6$, mean ± SD).

	AST (U/L)	MDA (μmol/L)
A	37.27 ± 7.46	14.86 ± 2.56
B	72.06 ± 14.38***	20.19 ± 2.23**
C	49.16 ± 15.29▲	16.10 ± 0.80▲
D	62.98 ± 13.14**	18.80 ± 2.05*
E	76.32 ± 15.64***	18.67 ± 1.71*
F	76.56 ± 17.48***	20.72 ± 1.59***
G	74.55 ± 18.02***	20.79 ± 2.09**

Notes: The contents of AST and MDA in the culture medium were determined after 3 h hypoxia and 2 h reoxygenation with different pre-treatments. (A) Control group, (B) H/R group, (C) H/R + *tribulosin* ($10\,nmol\cdot l^{-1}$) group, (D) PKCε inhibitor ($1\,nmol\cdot l^{-1}$) + *tribulosin* ($10\,nmol\cdot l^{-1}$) + H/R group, (E) PD98059 ($10\,\mu mol\cdot l^{-1}$) + TTSM ($100\,nmol\cdot l^{-1}$) + H/R group, (F) PKCε inhibitor ($1\,nmol\cdot l^{-1}$) + H/R group and (G) PD98059 ($10\,\mu mol\cdot l^{-1}$) + H/R group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control group; ▲$p < 0.05$ vs. I/R group ($n = 6$, $\bar{x} \pm s$).

with tribulosin, it increased. However, after pre-treatment with PKCε inhibitor or PD98059, survival rate also decreased, which has no significant deviation compared with H/R group, but has significant deviation with tribulosin group. This result shows that PKCε inhibitor and PD98059 can restrain cardioprotective effect of tribulosin (Figure 2).

2.3 Cardiac myocytes apoptosis

Apoptosis is a critical cellular event involved in the pathogenesis of myocardial I/R injury [11–13]. We have shown that tribulosin could reduce the expression of two key downstream effectors of cell death, cleave caspase-3 and bax, and increase bcl-2 with a resulting decrease in the number of apoptotic cardiomyocytes

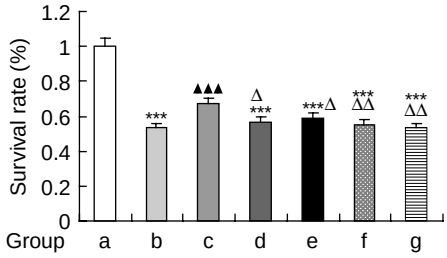

Figure 2. Effect of tribulosin on the survival ability of myocardial cell impaired by H/R. Cardiomyocytes were subjected to 3 h hypoxia and 2 h reoxygenation with different pre-treatments. Survival rate was detected. (a) Control group survival rate was 100%. (b) H/R group survival rate was 60.78%. (c) Tribulosin ($10\,nmol{\cdot}l^{-1}$) + H/R group survival rate was 76.47%. (d) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + tribulosin ($10\,nmol{\cdot}l^{-1}$) + H/R group survival rate was 64.71%. (e) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + tribulosin ($10\,nmol{\cdot}l^{-1}$) + H/R group survival rate was 66.67%. (f) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + H/R group survival rate was 62.75%. (g) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + H/R group survival rate was 60.78%. Each data point is the mean ± SEM from six independent experiments; $^{***}p < 0.001$, compared to control group; $^{\blacktriangle\blacktriangle}p < 0.01$, compared to H/R group; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared to tribulosin group; H/R, hypoxia/reoxygenation.

in isolated heart [6–8]. Thus, it is considered that the pre-treatment of hearts with tribulosin may elicit a protective effect against the cellular damage produced by subsequent I/R. This result suggested that tribulosin could interfere with the apoptotic signal pathway activated by H/R in cardiomyocytes.

Apoptosis was quantified by using fluorescent dye Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Viable cells were <3%. The percentages of apoptosis at every stage significantly increased in H/R group (Figure 3). Tribulosin reduced the occurrence of cardiac myocytes apoptosis induced by H/R. And the results showed that apoptotic cells treated with tribulosin were significantly lower than that of cells subjected to H/R. After pre-treatment with PKCε inhibitor or PD98059, cells exhibited higher apoptosis rate, and there was no significant difference compared with that in the H/R group.

2.4 Expression of phospho-PKCε

Mounting evidence indicates that PKC signaling pathway plays a key role in the protection of ischemic preconditioning (PC) [14–17]. PKC is a family of phospholipid-dependent serine/threonine kinases that regulate a wide variety of cellular functions. Each individual PKC isoform exhibiting a characteristic pattern possesses a unique biological function and redistribution. Activation of PKC has been shown to be an important signaling step in various biological processes, including the development of ischemic PC. Activation of PKC is usually associated with membrane translocation, and prolonged cellular exposure to PKC activators can cause PKC degradation or downregulation. This translocation occurs over the course of seconds to minutes [17]. It has been demonstrated that PKC-mediated cardioprotection is isoform specific and that the ε-isoform of PKC plays an essential role in the

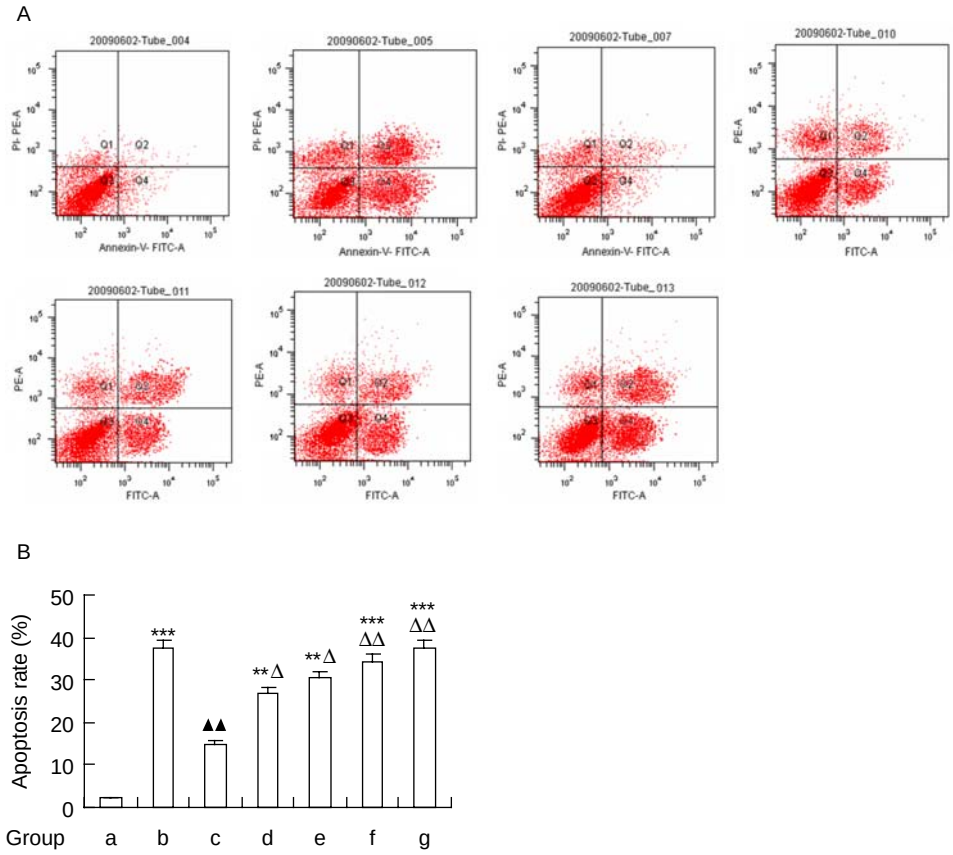

Figure 3. Effect of tribulosin on apoptosis of myocardial cell impaired by H/R. Cardiomyocytes were subjected to 3 h hypoxia and 2 h reoxygenation with different pre-treatments. (A) Apoptosis was quantified by FCM analysis after being stained with Annexin V and propidine iodide (PI). Horizontal axis shows Annexin V intensity and vertical axis represents PI staining. The lines divide each plot in four quadrants: viable cells (Annexin V−/PI−), early apoptosis (Annexin V+/PI−), late apoptosis (Annexin V+/PI+), and necrotic cells (Annexin V− /PI−). (a) Control group, (b) H/R group, (c) H/R+*tribulosin* ($10\,\text{nmol}\cdot\text{l}^{-1}$) group, (d) PKCε inhibitor ($1\,\text{nmol}\cdot\text{l}^{-1}$) + *tribulosin* ($10\,\text{nmol}\cdot\text{l}^{-1}$) + H/R group, (e) PD98059 ($10\,\mu\text{mol}\cdot\text{l}^{-1}$) + *tribulosin* ($10\,\text{nmol}\cdot\text{l}^{-1}$) + H/R group, (f) PKCε inhibitor ($1\,\text{nmol}\cdot\text{l}^{-1}$)+ H/R group, and (g) PD98059 ($10\,\mu\text{mol}\cdot\text{l}^{-1}$)+ H/R group. (B) Apoptosis rate. Each data point is the mean ± SEM from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control group; $^{\blacktriangle\blacktriangle}p < 0.01$ compared to H/R group; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared to tribulosin group; H/R, hypoxia/reoxygenation.

development of PC in myocardium [18]. PKCε is a member of the PKC family, which has been studied extensively in PC and tumorigenesis [19]. PKCε is believed to function as an antiapoptotic protein. Several studies have reported that the localization of PKCε is affected during apoptosis. Inhibition of this isoform completely blocks the delayed cardioprotection, supporting the concept that the activation of this signaling molecule is necessary for late PC [20]. It has been suggested that the targeted disruption of the PKCε gene leads to the loss of the cardioprotective effect of ischemic PC [21]. A PKCε inhibitory peptide efficiently inhibited Bcl-2 phosphorylation and augmented hydrogen peroxide-induced

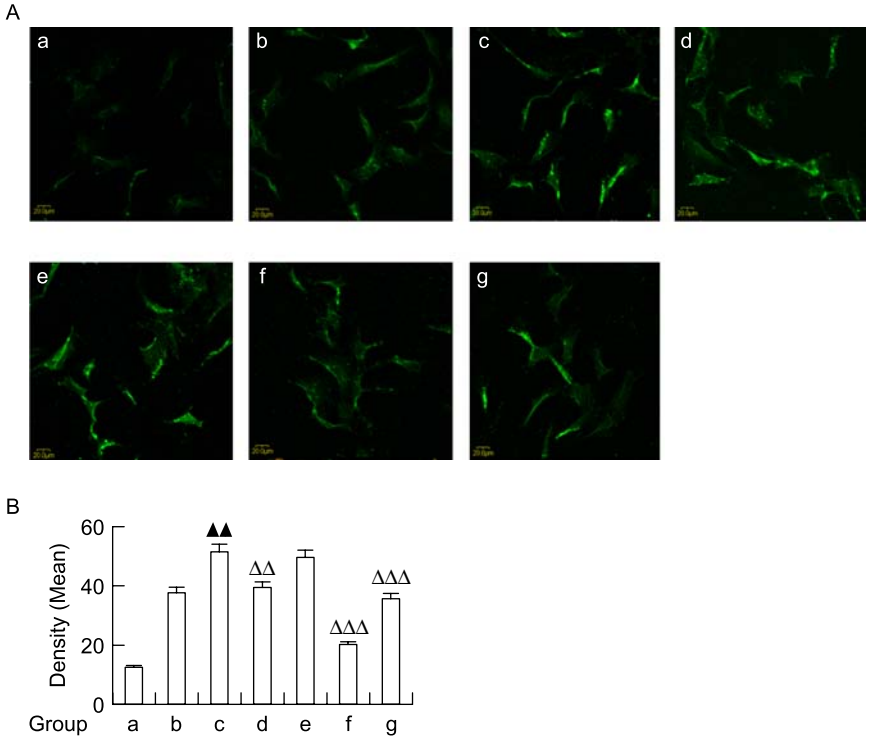

Figure 4. PKCε membrane translocation examined with a laser confocal scanning microscope (400 ×). Cardiomyocytes were subjected to 3 h hypoxia and 2 h reoxygenation with different pretreatments. (A) The expression of phospho-PKCε was examined with a laser confocal scanning microscope. (a) Control group, there was little phospho-PKCε expression; (b) H/R group, there was small amount of phospho-PKCε expression; (c) H/R + tribulosin ($10\,nmol{\cdot}l^{-1}$) group, phospho-PKCε expression was significantly increased; (d) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + tribulosin ($10\,nmol{\cdot}l^{-1}$) + H/R group, there was little phospho-PKCε expression; (e) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + tribulosin ($100\,nmol{\cdot}l^{-1}$) + H/R; (f) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + H/R; and (g) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + H/R. There was little phospho-PKCε expression in groups D, E, F, and G. (B) The mean density of fluorescence signal images. Each data point is the mean ± SEM from three independent experiments. $^{▲▲}p < 0.01$ compared to H/R group; $^{ΔΔ}p < 0.01$, $^{ΔΔΔ}p < 0.001$ compared to tribulosin group; H/R, hypoxia/reoxygenation.

apoptosis in a concentration-dependent manner in rat cardiomyocytes [22]. *In vivo* studies have demonstrated that during ischemic PC, activation of PKCε in cardiomyocytes protects against apoptosis, whereas targeted disruption of PKCε inhibits the beneficial effect of PC [23].

Translocation of PKCε is a property of activation. To investigate whether PKCε was involved in tribulosin-induced cardioprotection, we evaluated the expression of PKCε and its activity with a laser confocal scanning microscope (Figure 4). The result showed that there was little phospho-PKCε expression in control group, while there was small amount of phospho-PKCε expression in H/R group. In tribulosin group, phospho-PKCε expression was significantly increased, suggesting that tribulosin triggers PKCε activation. Furthermore, to test the role of PKCε in cardioprotection, we also introduced PKCε inhibitor before treated with H/R. Pretreatment with PKCε inhibitor inhibited the expression of phospho-PKCε. PD98059 had no obvious effect on phospho-PKCε

expression, which demonstrated that PKCε was crucial to protective effects.

2.5 *ERK1/2 nuclear translocation*

Although PKCε appears to play an essential role in the protective effect of tribulosin, the downstream signaling events of this specific isozyme remain largely unknown. Concomitant activation of several survival kinases creates important cross-talk. Among the signals, activation of the RAS/RAF/MEK/ERK pathway can promote cell proliferation and repress cell apoptosis. Members of the MAPK family have been implicated in survival signaling in response to I/R, oxidative stress, hypoxia, and anthracycline exposure [24]. A plausible target for PKC-mediated signaling events is the family of MAPKs [16,23]. The MAPK pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation, and apoptosis.

MAPK signaling pathways consist of a sequence of successively kinases that ultimately result in the dual phosphorylation and activation of terminal kinases such as p38, c-Jun N-terminal kinases, and ERKs. MAPKs are involved in multiple intracellular signaling cascades and are activated by the stimulation of a variety of cell surface receptors such as receptor tyrosine kinases, G protein-linked receptors, and cytokine receptors. On activation, MAPKs can phosphorylate and activate their target proteins and transcription factors, thereby initiating the transcription of new genes and the expression of new proteins [20]. Evidence indicates that ERK signaling is required to protect the myocardium from I/R injury *in vivo* [8,9]. In cardiac tissue, activation of ERK1/2 has been shown to be a necessary event during ischemic, and activation of ERK1/2 has been observed after brief ischemia. Inhibition of ERK signaling was demonstrated to increase daunomycin-induced apoptosis in cultured cardiomyocytes, whereas in a model of I/R in the intact heart, ERK1/2 activation was shown to attenuate the amount of apoptosis subsequent to reperfusion injury. ERK1/2 proteins are directly phosphorylated by MEK1/2 at both a threonine and an adjacent tyrosine residue. The activated ERK translocates into the nucleus and induces a series of transcription factors phosphorylation. The antiapoptotic mechanism of ERK is possibly involved in the activation of transcription factors, including AP-1, members of Bcl-2 family, CDK2, CDK4, nuclear factor-κ B, and so on [9].

In this study, to determine whether the phosphorylation of ERK1/2 in cardiac myocytes was caused by PKCε activation or tribulosin, we pretreated myocytes with PKCε inhibitor. The results showed that PKCε inhibitor blocked the enhanced phosphorylation of ERK1/2 and tribulosin-induced ERK1/2 by the activation of PKCε. To further elucidate the relation between ERK1/2 and PKCε, we pretreated myocytes with PD98059, a specific inhibitor of the ERK1/2 signaling pathway. As expected, PD98059 completely blocked PKCε-induced phosphorylation of ERK1/2, but pretreated with PD98059 had no significant effect on expression of PKCε. These results demonstrated that the activation of ERK1/2 was dependent on the activation of PKCε and ERK1/2, which were downstream signaling targets of PKCε in tribulosin-induced cardiac myocytes protection. To investigate whether ERK1/2 was involved in tribulosin-induced cardioprotection, we evaluated the expression of ERK1/2 and its activity with immunohistochemistry (Figure 5). After treating with *tribulosin*, phospho-ERK1/2 expression was obviously augmented, while ERK1/2 inhibitor PD98059 blocked the effects of *tribulosin*.

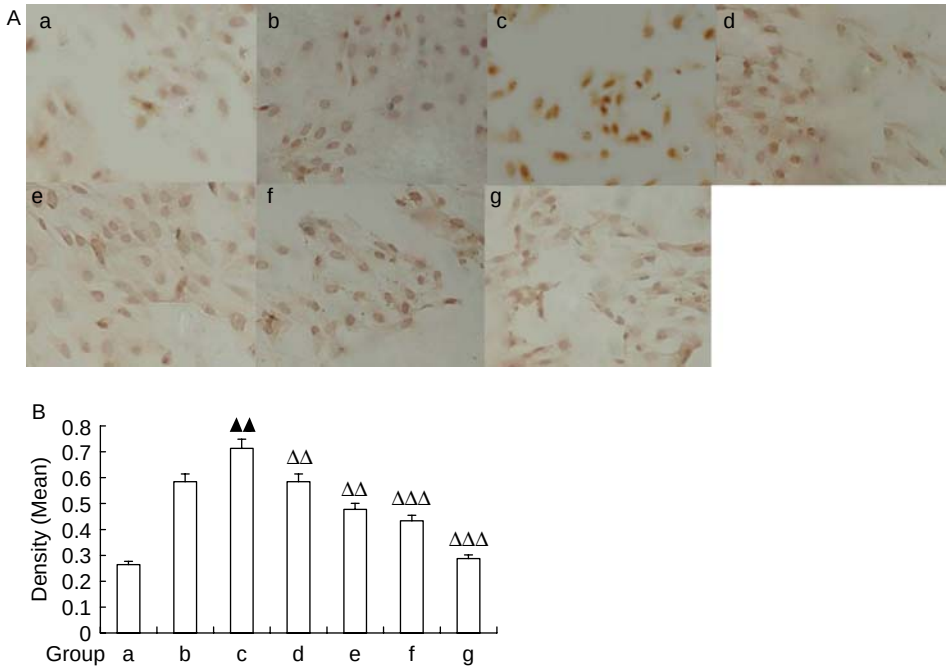

Figure 5. ERK1/2 nuclear translocation with immunohistochemistry (200 ×). Cardiomyocytes were subjected to 3 h hypoxia and 2 h reoxygenation with different pre-treatments. (A) The expression of phospho-ERK1/2 was examined with immunohistochemistry. (a) Control group, (b) H/R group, (c) *tribulosin* (10 nmol·l^{-1}) + H/R group, (d) PKCε inhibitor (1 nmol·l^{-1}) + *tribulosin* (10 nmol·l^{-1}) + H/R group, (e) PD98059 (10 μmol·l^{-1}) + *tribulosin* (10 nmol·l^{-1}) + H/R group, (f) PKCε inhibitor (1 nmol·l^{-1}) + H/R group, and (g) PD98059 (10 μmol·l^{-1}) + H/R group. After treated with *tribulosin*, phospho-ERK1/2 expression was obviously augmented, while ERK1/2 inhibitor PD98059 blocked the effects of *tribulosin*. (B) The mean density. Each data point is the mean ± SEM from three independent experiments. $^{▲▲}p < 0.01$, compared to H/R group; $^{ΔΔ}p < 0.01$, $^{ΔΔΔ}p < 0.001$ compared to tribulosin group; H/R, hypoxia/reoxygenation.

2.6 Western blot analysis of the expression of PKCε and ERK1/2

To further investigate the relation of PKCε and ERK1/2 involved in *tribulosin*-induced cardioprotection, we evaluated the expression of PKCε and ERK1/2 by Western blotting analysis (Figure 6). After treating with *tribulosin*, phospho-PKCε and ERK1/2 were augmented. While PKCε inhibitor blocked the effects of *tribulosin*, PD98059 had no significant effect on the expression of PKCε.

In conclusion, tribulosin has protective effect on cardiac myocytes impared by H/R, the mechanism of which depended on the activation of PKCε. When treated with tribulosin, which can activate PKCε, and PKCε can activate ERK1/2, then ERK1/2 activates the downstream signal further to reduce the cardioprotective effect. Tribulosin suppresses apoptosis during H/R via PKCε and ERK1/2 signaling pathway.

3. Experimental

3.1 Material

3.1.1 Source and identification

Aerial parts of *T. terrestris* were collected in August 2007 in the suburb of Baicheng city of Jilin Province, China, and identified by Professor Minglu Deng

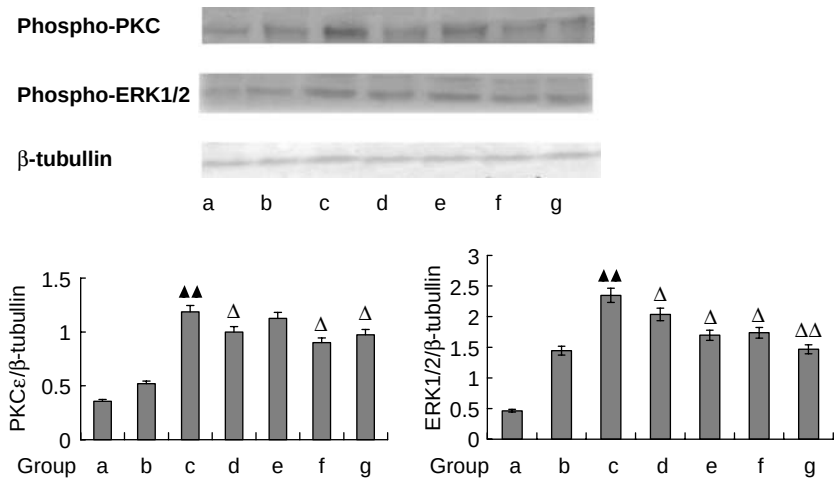

Figure 6. Effect of tribulosin on PKCε and ERK1/2 expression. Cardiomyocytes were subjected to 3 h hypoxia and 2 h reoxygenation with different pre-treatment. (A) The expression of phospho-PKCε and phospho-ERK1/2 was examined with Western blotting. (a) Control group, (b) H/R group, (c) *tribulosin* ($10\,nmol{\cdot}l^{-1}$) + H/R group, (d) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + *tribulosin* ($10\,nmol{\cdot}l^{-1}$) + H/R group, (e) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + *tribulosin* ($10\,nmol{\cdot}l^{-1}$) + H/R group, (f) PKCε inhibitor ($1\,nmol{\cdot}l^{-1}$) + H/R group and (g) PD98059 ($10\,\mu mol{\cdot}l^{-1}$) + H/R group. After treated with *tribulosin*, phospho-PKCε and ERK1/2 were augmented. While PKCε inhibitor blocked the effects of *tribulosin*, PD98059 had no significant effect on the expression of PKCε. (B) Each data point is the mean ± SEM from three independent experiments. $^{\blacktriangle\blacktriangle}p < 0.01$, compared to H/R group; $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared to tribulosin group; H/R, hypoxia/reoxygenation.

(Changchun University of Traditional Chinese Medicine, Changchun, China).

3.1.2 *Drugs and reagents*

Tribulosin and GSTT were extracted and isolated by the *College of Chemistry, Jilin University*. Tribulosin was obtained as a white powder with molecular weight 1150, whose purity exceeded 99.99%. It was dissolved in dimethyl sulfoxide (DMSO) for use. GSTT is a yellow powder, whose purity exceeded 90%. *Tribulosin* and GSTT were dissolved in Iscove's Modified Dulbecco's Medium (IMDM, from Invitrogen GIBCO, Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Yuanhengjinma (Beijing, China) and trypsin from Biotech (Changchun, China). AST, MDA, and Coomassie Blue were all purchased from Jiancheng Biotechnology (Nanjing, China). PD98059 (ERK1/2 inhibitor), 5-bromo-2′-deoxyuridine (BrdU), and anti-β-tubulin were purchased from Sigma Chemicals (St. Louis, MO, USA). PKC (ε inhibitor) and anti-PKCε and anti-ERK1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies to goat or rabbit were obtained from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China). Immunohistochemistry kit was purchased from Bios (Beijing, China). All other chemicals used in the present study were of analytical grade.

Hypoxia solution ($mmol \cdot l^{-1}$): Na_2HPO_4 0.9, $NaHCO_3$ 6.0, $CaCl_2$ 1.8, $MgSO_4$ 1.2, natrium lacticum (SL) 40, HEPES 20.0, NaCl 98.5, KCl 10.0, and pH 6.8.

Reoxygen solution ($mmol \cdot l^{-1}$): Na_2HPO_4 0.9, $NaHCO_3$ 20.0, $CaCl_2$ 1.8, $MgSO_4$ 1.2, glucose 55.0, HEPES 20.0, NaCl 129.5, and pH 7.4.

3.1.3 Animals

We followed the Guiding Principles for the Care and Use of Laboratory Animals approved by Animal Regulations of National Science and Technology Committee. Wistar rats of 1–3 days old were purchased from the Center of Experimental Animals of Jilin University. The animals' certificate number was SCXK(JI)2007-0003.

3.2 Primary cultures of cardiac myocytes

Hearts were quickly removed from neonatal rats and washed with cold D-Hank's buffered saline, minced, and incubated with 0.1% trypsin at 37°C for 5 min. Then fresh 0.1% trypsin solution was added and the incubation procedure was repeated until the tissue was totally digested. The supernatant was collected and centrifuged at 400*g* for 10 min. The obtained cell pellet was resuspended in IMDM containing 15% FBS, then plated in a culture flask and incubated for at least 90 min at 37°C in a 5% CO_2 incubator. Fibroblasts adhered to the culture flask surface while the cardiac myocytes remained unattached. The latter were then diluted to 3.0×10^8/liter, plated in 24-well plates for immunocytochemistry assay, and diluted to 5.0×10^8/liter, plated in 100-ml glass flasks for flow cytometry (FCM) and Western blotting assay. BrdU at a final concentration of $0.1\ \text{mmol} \cdot \text{l}^{-1}$ was added during the first 48 h to inhibit the proliferation of fibroblasts. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and medium was changed after 48 h.

3.3 Protocol of H/R injury

H/R injury model was prepared as Ref. [7]. Cardiac myocytes cultured for 72 h were serum-free for another 24 h before use. Cardiac myocytes were placed in hypoxic solution equilibrated for 30 min with 95% N_2 and 5% CO_2, and the oxygen partial pressure was then lowered to 7 kPa, then closed tight in 37°C over the experimental time. After 3 h of hypoxia, the cells were subjected to reoxygenation by changing the medium into reoxygenation solution within a CO_2 incubator (containing 95% O_2 and 5% CO_2) for 2 h.

The cultured cardiac myocytes were randomly divided into seven groups:

1. *Group A* (Control group): Cardiac myocytes were kept in reoxygenation solution within a CO_2 incubator (containing 95% O_2 and 5% CO_2) for 5 h.
2. *Group B* (H/R group): Cardiac myocytes were exposed to 3 h of hypoxia followed by 2 h of reoxygenation.
3. *Group C* (Tribulosin + H/R group): Cardiac myocytes were pretreated with tribulosin ($10\ \text{nmol} \cdot \text{l}^{-1}$) for 0.5 h followed by H/R.
4. *Group D* (PKCε inhibitor + tribulosin + H/R group): Cardiac myocytes were pretreated with PKCε inhibitor ($1\ \text{nmol} \cdot \text{l}^{-1}$) for 0.5 h, then tribulosin ($10\ \text{nmol} \cdot \text{l}^{-1}$) for 0.5 h followed by H/R.
5. *Group E* (PD98059 + tribulosin + H/R group): Cardiac myocytes were pretreated with PD98059 ($10\ \mu\text{mol} \cdot \text{l}^{-1}$) for 0.5 h, then tribulosin ($10\ \text{nmol} \cdot \text{l}^{-1}$) for 0.5 h followed by H/R.
6. *Group F* (PKCε inhibitor + H/R group): Cardiac myocytes were pretreated with PKCε inhibitor ($1\ \text{nmol} \cdot \text{l}^{-1}$) for 1 h followed by H/R.
7. *Group G* (PD98059 + H/R group): Cardiac myocytes were pretreated with PD98059 ($10\ \mu\text{mol} \cdot \text{l}^{-1}$) for 1 h followed by H/R.

3.4 Survival rates

The survival rates of cardiac myocytes were determined by the methyl thiazolyl tetrazolium (MTT) assay. Cells were

seeded into 96-well plates and exposed to various treatments. After treatments, 10 μl of MTT ($5\,g \cdot l^{-1}$) was added into each well, and the cells were incubated for another 4 h at 37°C. Afterwards, the media were discarded, and DMSO (150 μl) was added for the dissolution of formazan crystals. The absorbance of each well was read at recording absorbance 490 nm with microplate reader. Cell viability was expressed as a percentage of the control.

3.5 Biochemical parameters

After various treatments, the amount of AST and MDA released into the medium was determined using a diagnostic kit.

3.6 Cardiac myocytes apoptosis rate

Annexin V-FITC/PI staining was performed after 2 h reoxygenation according to the manufacturer's instructions. After digesting with 0.25% trypsin, cells were gathered and rinsed with ice-cold phosphate buffer saline (PBS) and then resuspended in 400 μl of binding buffer. Annexin V stock solution (10 μl) was added to the cells and incubated for 15 min. The cells were then further incubated with 5 μl PI and were immediately analyzed on a FCM.

3.7 Protein fractionation and Western blot

Myocytes were lysed at 4°C with ice-cold lysis buffer containing all-in-one (phosphatase inhibitors mixtures, Beyotime Biotechnology, Nantong, Jiangsu, China) and phenylmethyl sulfonylfluoride (final concentration at $10\,mmol \cdot l^{-1}$) for 30 min. Cell lysates were centrifuged at 12,000*g* for 10 min and protein concentrations in the supernatants were quantitated with Coomassie Blue. The protein sample (20 μg) was separated by 12% denaturing SDS–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After electrophoresis, the proteins were transferred to nitrocellulose membrane by electrophoretic transfer system. The membranes were blocked in 5% skimmed milk (or bovine serum albumin, BSA) in PBS for 2 h, and then incubated with primary antibody (anti-PKCε and anti-ERK1/2 antibodies at 1:200 dilution, anti-β-tubulin at 1:5000 dilution) overnight at 4°C. After washing with PBS–Tween-20, the membranes were incubated for 2 h in horseradish peroxidase-conjugated rabbit anti-goat antibodies (1:1000 dilution) for 2 h. After washing with PBS-T, the bands were detected by 3,3′-diaminobenzidine, and then analyzed with Tanon GIS gel image processing system.

3.8 Expression of PKCε

Myocytes in the 24-well plate were washed with PBS at 37°C, then formalin was fixed for 30 min with 4% paraformaldehyde, and then washed with distilled water. The membrane was ruptured with 0.3% Triton X-100 for 15 min. Myocytes were incubated with 1% BSA for 30 min to block the reaction, then washed. Anti-phospho-PKCε diluted by 1:50 was added dropwise at 300 μl per pore and allowed to stay over 12 h 4°C. After washing with PBS, rabbit anti-goat antibody labeled with FITC by valency at 1:50 was added dropwised and incubated at 37°C for 30 min. The cells were then further incubated with 300 μl PI before washing with PBS and were immediately analyzed on a laser confocal scanning microscope.

3.9 Immunohistochemistry detection of ERK1/2

Myocyte slides were washed with PBS at 37°C, and then fixed with 4% paraformaldehyde for 30 min. After washing steps, the expression of phospho-ERK1/2 was detected using immunohistochemistry methods.

3.10 Statistical analysis

All data are expressed as mean ± standard deviation (SD). Means between the groups were evaluated by the one-way analysis of variance followed by Schiff's test. A p value <0.05 was considered as statistically significant.

Acknowledgments

The authors acknowledge Dr Katori for his help in writing the paper. The authors also acknowledge the simple monomers separation of GSTT by the *College of Chemistry, Jilin University*. This work was financially supported by grants (Nos 30472020 and 30672654) from the National Natural Science Foundation of China.

References

[1] Y.G. Zhang, H.Y. Song, Y. Li, S. Zhang, and S.J. Yang, *Chin. Pharmacol. Bull.* **26**, 421 (2010).
[2] W. Sun, H. Li, and S.J. Yang, *J. Asian Nat. Prod. Res.* **10**, 39 (2008).
[3] H. Li, S. Zhang, Z. Shi, and S.J. Yang, *J. Jilin Univ. Med. Ed.* **34**, 927 (2008).
[4] S. Zhang, H. Li, Z.R. Wei, L Liang, and S.J. Yang, *Chin. Tradit. Herb. Drugs* **41**, 34 (2010).
[5] S. Zhang, H. Li, H. Xu, and S.J. Yang, *Acta. Pharm. Sin.* **45**, 31 (2010).
[6] S. Zhang, R.J. Yang, H. Li, Z.Y. Yin, H.Y. Zhou, Y.R. Jin, X.W. Li, and S.J. Yang, *Chem. Res. Chin. Univ.* **26**, 915 (2010).
[7] S. Zhang, H. Li, and S.J. Yang, *Chin. Pharmacol. Bull.* **26**, 208 (2010).
[8] S. Zhang, H. Li, and S.J. Yang, *Acta. Pharmacol. Sin.* **31**, 671 (2010).
[9] M. Takashi and R. Anthony, *J. Mol. Cell Cardiol.* **38**, 63 (2005).
[10] M. Patrick, E.B. Kim, C.P. Marie, and P. Philippe, *FEBS J.* **276**, 1667 (2009).
[11] R.S. Neel, T.C. Richard, F. Jun, L. Yuhong, B. Cesario, M.H. Eszter, S. Csaba, L.S. Gregory, and W.S. Frank, *J. Thorac. Cardio-vasc. Surg.* **138**, 977 (2009).
[12] R.S. Neel, T.C. Richard, F. Jun, L. Yuhong, B. Cesario, M.H. Eszter, S. Csaba, and W.S. Frank, *Eur. J. Cardiothorac. Surg.* **33**, 906 (2008).
[13] Y.L. Wang, C.Y. Wang, B.J. Zhang, and Z.Z. Zheng, *Mol. Biol. Rep.* **36**, 365 (2009).
[14] Z.H. Shao, W.T. Chang, K.C. Chan, K.R. Wojcik, C.W. Hsu, C.Q. Li, J. Li, T. Anderson, Y. Qin, L.B. Becker, K.J. Hamann, and T.L. Vanden, *Am. J. Physiol. Heart Circ. Physiol.* **292**, 1995 (2007).
[15] Y. Qiu, P. Ping, X.L. Tang, S. Manchikalapudi, A. Rizvi, J. Zhang, H. Takano, W.J. Wu, S. Teschner, and R. Bolli, *J. Clin. Invest.* **101**, 2182 (1998).
[16] A.M. Kabir, J.E. Clark, M. Tanno, X. Cao, J.S. Hothersall, S. Dashnyam, D.A. Gorog, M. Bellahcene, M.J. Shattock, and M.S. Marber, *Am. J. Physiol. Heart Circ. Physiol.* **291**, 1893 (2006).
[17] L.R. Seth, G. Jyothi, G.R. Valentin, and W.W. Jeffery, *Biophys. J.* **80**, 2140 (2001).
[18] P. Ping, C. Song, J. Zhang, Y. Guo, X. Cao, R.C. Li, W. Wu, T.M. Vondriska, J.M. Pass, X.L. Tang, W.M. Pierce, and R. Bolli, *J. Clin. Invest.* **109**, 499 (2002).
[19] B. Alakananda and S. Usha, *Cell Signal.* **19**, 1633 (2007).
[20] R.C. Li, P. Ping, J. Zhang, W.B. Wead, X.N. Cao, J.M. Gao, Y.T. Zheng, S. Huang, J.H. Han, and R. Bolli, *Am. J. Physiol. Heart Circ. Physiol.* **279**, 1679 (2000).
[21] R.B. Grant, N.C. Eric, and M.R. Daria, *Pharmacol. Res.* **55**, 523 (2007).
[22] K.M. Lawrence, A.M. Kabir, M. Bellahcene, S. Davidson, X.B. Cao, J. McCormick, R.A. Mesquita, C.J. Carroll, A. hanalaris, P.A. Townsend, M. Hubank, A. Stephanou, R.A. Knight, M.S. Marber, and D.S. Latchman, *FASEB J.* **19**, 831 (2005).
[23] M. Rabban, S. Tushi, S.D. Naranjan, and S.T. Paramjit, *Am. J. Physiol. Heart Circ. Physiol.* **291**, 854 (2006).
[24] O.F. Bueno and J.D. Molkentin, *Circ. Res.* **91**, 776 (2002).