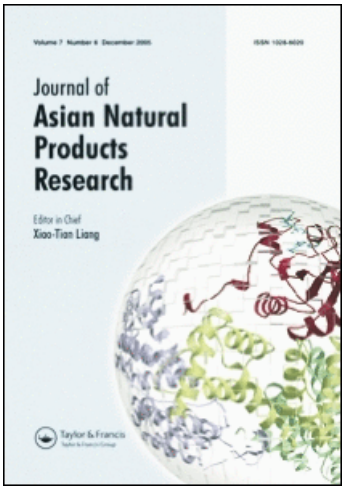

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Sun, WEI , Li, HONG and Yang, SHI-JIE(2008) 'A triterpene saponin from *Tribulus terrestris* attenuates apoptosis in cardiocyte via activating PKC signalling transduction pathway', Journal of Asian Natural Products Research, 10: 1, 39 — 48
To link to this Article: DOI: 10.1080/10286020701275846
URL: http://dx.doi.org/10.1080/10286020701275846

Journal of Asian Natural Products Research, Vol. 10, No. 1, January 2008, 39–48

A triterpene saponin from *Tribulus terrestris* attenuates apoptosis in cardiocyte via activating PKC signalling transduction pathway

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(Received 5 July 2006; revised 14 December 2006; in final form 20 December 2006)

The present study was conducted to examine the role of hecogenin-3-*O*-β-D-glucopyranosyl(1 → 4)-β-D-galactopyranoside (**1**), which is a triterpene saponin of *Tribulus terrestris* in cardiocytes during chemical hypoxia-ischaemia *in vitro*. Neonatal rat ventricular myocytes were isolated by collagenase digestion and treated with NaCN for 12 h. Cell apoptosis was defined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and flow cytometry. [Ca^{2+}] was measured by confocal microscopy. There was a marked increase in the expression of the anti-apoptotic protein, Bcl-2, by NaCN. This change was increased by the saponin **1**. PKCε protein contents were increased in the cardiocyte membrane fraction in response to NaCN. PKCε activation was augmented by the saponin **1**. Inhibition of PKCε with inhibitory peptide prevented Bcl-2 expression. Moreover, the saponin attenuated the apoptosis in cardiocyte in response to NaCN. It is therefore suggested that the saponin **1** may play a role in cardiocyte survival *via* PKCε and Bcl-2.

Keywords: Hecogenin-3-*O*-β-D-glucopyranosyl(1 → 4)-β-D-galactopyranoside; Hypoxia/ischaemia; [Ca^{2+}]; Bcl-2; PKC; Signal transduction

1. Introduction

Cardiocyte apoptosis contributes to functional deterioration in ischaemia/hypoxia cardiomyopathies. Unlike necrosis, apoptosis is a tightly controlled, energy-dependent process that is mediated by discrete intracellular signals. It is characterised by cell shrinkage, nuclear condensation and DNA fragmentation, and in the latter stages the cell membrane devolves into neatly packaged vesicles or apoptotic bodies, that are taken up by neighbouring cells [1,2]. Not surprisingly, many important stress-activated signal transduction pathways within the heart have emerged as powerful regulators of the apoptotic process. On a molecular level, apoptosis is associated with changes in the expression and phosphorylation states of numerous pro-and anti-apoptotic proteins such as Bcl-2, BAD and Bax [3,4] that subsequently lead to the activation of downstream apoptotic effects.

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Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2008 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020701275846

Regulating the levels of pro- and anti-apoptotic proteins may be a way in which the damage from ischaemia and hypoxia is propagated, and PKC may regulate this process. PKCε activity may also regulate the expression of anti-apoptotic Bcl-2. Therefore, PKCε may also exert its anti-apoptotic activity by regulating the Bcl-2 family of proteins, as well. The activity and ratio of the Bcl-2 family of proteins may influence the outcome of an ischaemic and hypoxia insult. And translocation of PKC isozymes from the cytosolic to the membrane may result in an alteration in intracellular Ca^{2+} concentration [5].

Our previous work showed that gross saponin *Tribulus terrestris* had the effect of lowering blood pressure and stimulating anti-hypoxia, sheeting blood vessels, lowering cholesterol, and inhibiting fat deposition on arteries, myocardium and liver. It also inhibited atherosclerotic occurrence and development, protecting the isolated heart from the ischaemia/reperfusion (I/R) injury. But it is hard to define the biological target of drug action, so we investigated biologic activity of hecogenin-3-*O*-β-D-glucopyranosyl(1 → 4)-β-D-galactopyranoside (**1**), which is one of the purified products of gross saponin *Tribulus terrestris*. However, no studies have addressed the effect of **1** on cardiocyte apoptosis and the interference of the saponin with signalling pathways. The aims of this study were, therefore, to determine whether the saponin can attenuate apoptosis induced by NaCN in cultured neonatal rat cardiocytes *in vitro* and to identify the molecular mechanisms that may be responsible for the putative effects of the saponin **1** (figure 1).

2. Results and discussion

2.1 Cell viability

In vitro experiments were performed to determine whether the saponin **1** could antagonise NaCN-induced cardiocyte death. Cardiocyte survival rate was evaluated by Trypan blue exclusion (figure 2), whereas cardiocyte apoptosis was measured with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and flow cytometry. The survival rate of cardiocytes decreased in NaCN medium, from 91.17 ± 3.3% to 68.15 ± 7.9% ($n = 6$, $P < 0.001$ *vs* untreated cells), and the survival rate of cardiocytes of the saponin **1** (30, 100 μg/ml) mediated by NaCN were 77.23 ± 5.6% and 80.48 ± 6.7%, respectively ($n = 6$, $P < 0.001$ *vs* exposed to NaCN only).

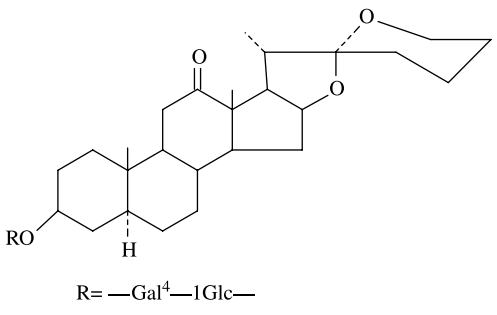


Figure 1. Chemical structure of the saponin **1**.

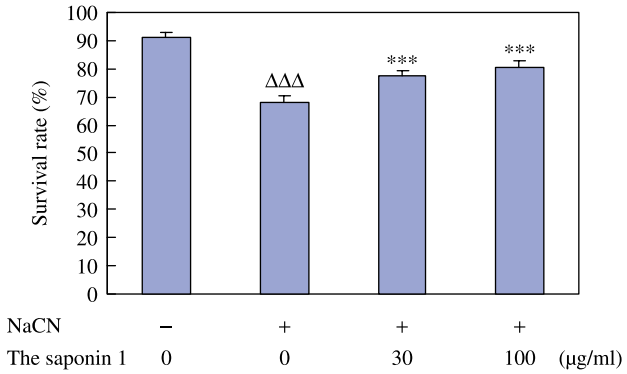


Figure 2. Cell viability measurement using Trypan blue assay after 12 h of treatment with 3 mmol/L NaCN and 30, 100 μg/ml of the saponin **1**. Data are mean ± SD. $n = 6$. $^{\Delta\Delta\Delta}P < 0.001$, *vs* untreated cells; $^{***}P < 0.001$, *vs* exposed to NaCN only.

2.2 Quantification of apoptotic cardiocytes

TUNEL assay showed that apoptotic cells had condensed nucleoli (figure 3B). The quantitative results of counting TUNEL-positive nucleoli correlated well with those of flow cytometry. Counting of TUNEL-positive cells under the microscope with the naked eye has more subjective components; thus we only presented the percentage of cell apoptosis obtained with flow cytometry.

Twelve hours of ischaemia mediated by NaCN produced significantly higher apoptosis (16.65 ± 0.12%, $n = 8$; figure 3B), compared with untreated cells (0.35 ± 0.11%, $n = 8$; figure 3A) ($P < 0.05$). The saponin **1** (30, 100 μg/ml) mediated by NaCN significantly decreased apoptotic rate (7.31 ± 0.42, figure 3C; 1.84 ± 0.27, figure 3D; $n = 8$) ($P < 0.05$), compared with exposure to NaCN only.

2.3 Intracellular Ca^{2+} fluorescence signal in cardiocytes

Cells were loaded with the fluorescent dye Fluo-3/AM as described above. Fluorescence intensity and distribution of the Ca^{2+} fluorescence signal in a single cell were measured by LSCM at 488 nm excitation wavelength. The differences observed in the Ca^{2+} fluorescence intensity and distribution can be further analysed using image analysis software of Leica Confocal Microscope Systems. The plot shows fluorescence intensity along an axis drawn through the cell; fluorescence intensity was automatically calculated by an internal calcium standard. The total fluorescence intensity of Ca^{2+} in the untreated cells area (figure 4A) and exposed to NaCN only area (figure 4B) were 280.18 ± 48.09 and 497.12 ± 103.70, respectively ($n = 12$, $P < 0.001$). These results clearly indicate that NaCN can increase the intracellular free Ca^{2+} concentration. The saponin (30, 100 μg/ml) mediated by NaCN significantly decreased the intracellular free Ca^{2+} concentration (323.73 ± 68.10, figure 4C; 345.24 ± 49.23, figure 4D; $n = 12$) ($P < 0.05$) compared with exposed to NaCN only.

2.4 Involvement of Bcl-2 in the cardioprotective effects of the saponin 1

In order to investigate the origins of these changes in the resistance to apoptosis induced by NaCN after treatment with the saponin and εV1-2 (PKCε inhibitor), an analysis of Bcl-2

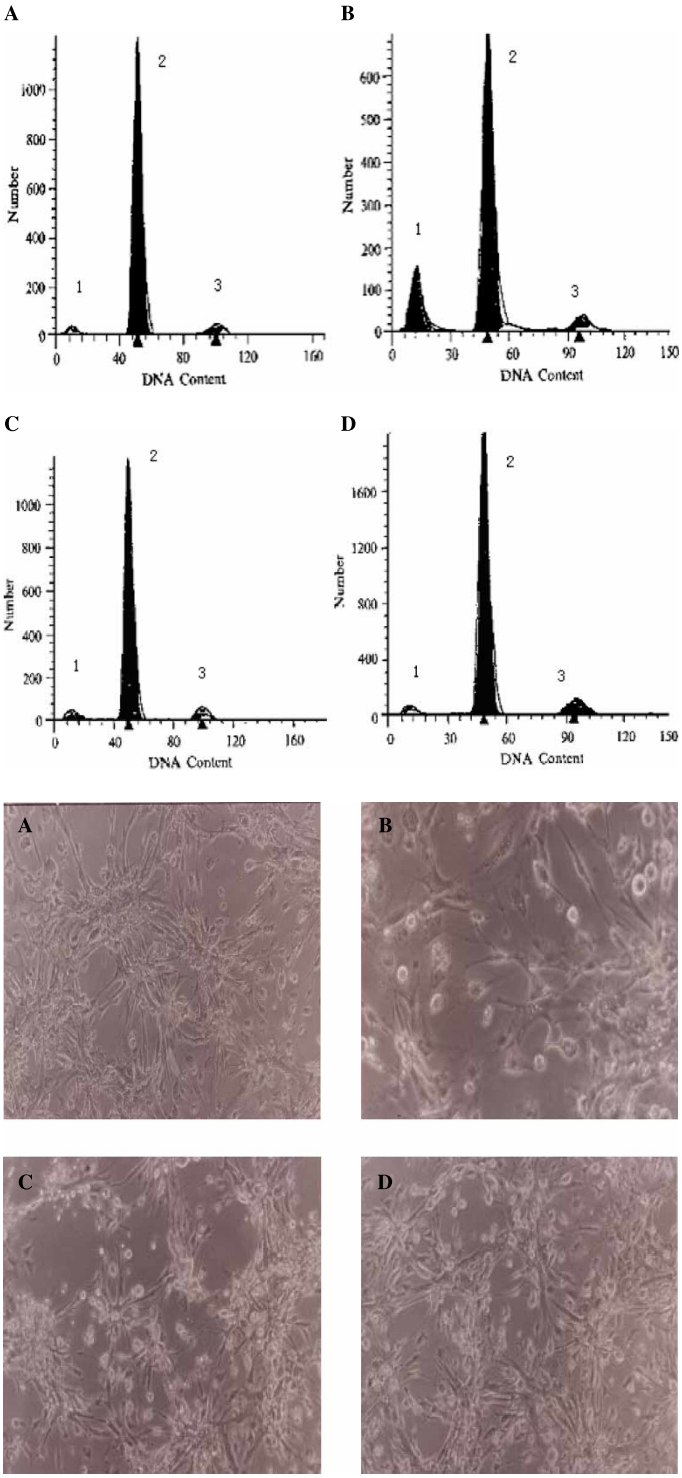


Figure 3. The saponin **1** attenuates apoptosis. Cardiocyte apoptosis was analysed by two complementary techniques. Flow cytometry provided quantitative results that were consistent with TUNEL. NaCN (3 mmol/L, 12 h) induced 16.65% apoptosis (B). The saponin **1** (30 μg/ml, C; 100 μg/ml, D) significantly reduced apoptosis compared with exposed to NaCN only ($P < 0.05$, $n = 8$).

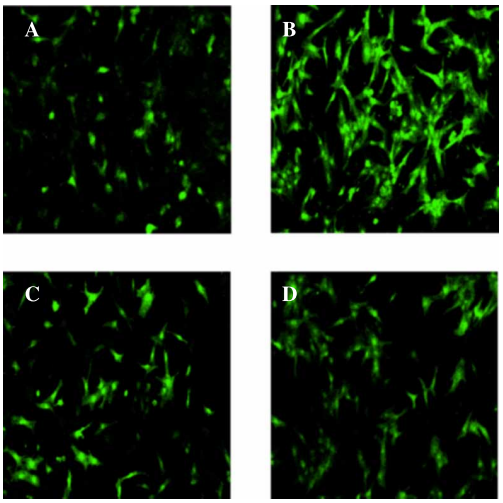


Figure 4. Calcium fluorescence signal images of cardiocytes detected by LSCM. Cardiocytes were grown in a sterile Petri dish. Relative levels of intracellular free calcium are indicated by the fluorescence intensity of the Fluo-3–Ca^{2+} complex detected by LSCM at 488 nm excitation wavelength.

protein, known to be implicated in apoptosis, was carried out as shown in figure 5. Bcl-2 protein levels were sensitive to drug treatment, the saponin (100 μg/ml, 12 h) mediated by NaCN enhanced Bcl-2 levels, whereas εV1-2 decreased Bcl-2 expression.

2.5 Involvement of total and phosph–PKCε in the cardioprotective effects of the saponin 1

We investigated the possible involvement of PKCε in the cardioprotective activity of the saponin against NaCN-induced cardiocytes apoptosis. From LSCM we can see that the fluorescence intensity of PKCε in the untreated cells protein area (figure 6A) and exposed to NaCN only area (figure 6B) were 382 ± 32.36 and 492 ± 44.94, respectively ($n = 12$, $P < 0.01$), and the saponin **1** (30, 100 μg/ml) mediated by NaCN significantly increased PKCε concentration (932 ± 17.89, figure 6C; 1188 ± 92.84, figure 6D) ($n = 12$, $P < 0.05$, $P < 0.01$) compared with exposure to NaCN only.

Cardiocytes injured by NaCN led to detectable changes in PKCε and phosph–PKCε partitioning in the particulate fractions at 12 h. Persistent saponin stimulation (100 μg/ml, 12 h) led to no significant difference of reduction in the abundance of PKCε and phosph–PKCε in the cytosolic fraction; but PKCε and phosph–PKCε abundance in the particulate

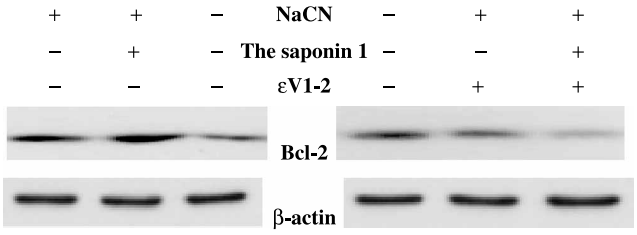
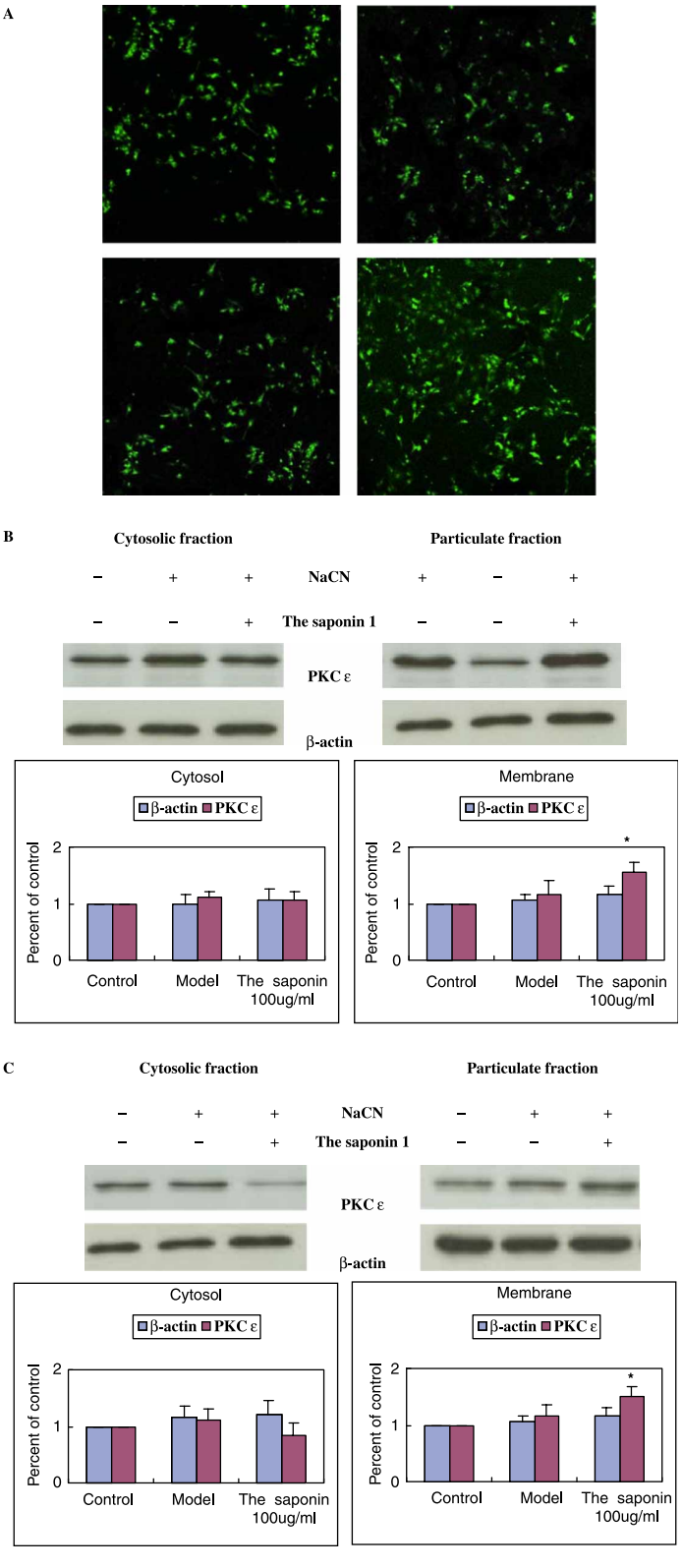


Figure 5. Effects of the saponin **1** on Bcl-2 protein levels was assessed with Western blot. The percentage of Bcl-2 in the cardiocytes was shown. Data are mean ± SD, $n = 6$, $*P < 0.05$ *vs* exposed to NaCN only.



fraction is increased then exposed to NaCN only. This result indicates a potential role of PKCε and phosph–PKCε for the saponin stimulation effects in cardiocytes. Western blot for PKCε and phosph–PKCε detected in the soluble and the particulate fractions after various teams in cardiocytes. For an equivalency of loading, β-actin was used as control for the cytosolic fraction and the particulate fraction, respectively.

The biochemical effects of NaCN is related to the inhibition of the mitochondrial respiratory chain, including the involvement of apoptosis, which primarily depends on NaCN-induced conversion of haemoglobin into methaemoglobin, releasing the blockade of cytochrome *c* oxidase by high-affinity binding of cyanide as cyanmethaemoglobin. For the basic pathophysiological process of clinic myocardial ischaemia and hypoxia by mitochondria damage, we characterised the effects of hecogenin-3-*O*-β-D-glucopyranosyl (1 → 4)-β-D-galactopyranoside (**1**) on NaCN-induced cardiocyte apoptosis in cultured neonate rat.

The present study elucidates the biological effects of the saponin **1** on cardiocytes apoptosis. The saponin **1** could increase the survival rate of cardiocytes under hypoxic circumstances and reduce apoptosis percentage. These results support the notion that the saponin can protect against cardiocyte apoptosis induced by NaCN *in vitro*. The cardioprotection of the saponin is probably caused by direct or indirect degression of intracellular free Ca^{2+} concentration and the increase in Bcl-2 protein levels. This indicates that the saponin could inhibit the opening of mitochondrial transition pores, thus reducing the release of apoptosis-induced proteins. Then it could inhibit the apoptosis process by stopping the caspase cascade through modulating the protein expression of the Bcl-2 family. Besides all that is mentioned above, it could maintain the calcium balance (the calcium overload could induce the apoptosis of cardiocytes).

Considerable data has demonstrated that PKCε might be important for cardiac protection. The isozyme selective peptide inhibitors and activators were used to test for the abilities of different PKCs to protect cardiocytes from ischaemic damage in a variety of models, including isolated neonatal and adult cardiocytes and intact hearts [6]. It was found that ischaemic preconditioning activates PKCε [7,8], as well as treatments that mimic preconditioning such as arachidonic acid [9], exposure to low amounts of ethanol, or by directly applying the PKCε agonist peptide. Moreover, selective inhibition of PKCε during preconditioning [4] results in loss of cardiac protection, indicating that PKCε activation is required and sufficient to mediate cardiac protection from ischaemic damage. Our study shows that the saponin stimulation does not lead to significant difference of reduction in the abundance of PKCε and phosph–PKCε in the cytosolic fraction; but PKCε and phosph–PKCε abundance in the particulate fraction is increased more than that exposed to NaCN only. This result indicates a potential role of PKCε and phosph–PKCε for MSTT/the saponin **1** stimulation effects in cardiocytes. It shows that **1** may be a PKCε activator or be mimicking preconditioning.

PKCε activation was found to induce the phosphorylation of BAD indirectly, which is a pro-apoptotic Bcl-2-related protein. So it renders BAD unable to participate in apoptosis

Figure 6. (A) PKC epsilon fluorescence signal images of cardiocytes detected by LSCM. Cells were grown in the sterile Petri dish. Relative levels of PKC epsilon are indicated by the fluorescence intensity of the FITC–PKCε complex detected by LSCM at 525 nm excitation wavelength. (B, C) Effects of the saponin **1** on the membrane translocation of PKCε and P-PKCε mediated by NaCN. The translocation was assessed with Western blot. The percentage of PKCε and P-PKCε in the cytosolic fraction and particulate fraction was shown. Data are mean ± SD, $n = 6$, $*P < 0.05$ *vs* exposed to NaCN only.

[10,11]. This may help explain how PKCε is cardioprotective during preconditioning. PKCε may also regulate the expression of anti-apoptotic Bcl-2. Our experimental results showed the changes in Bcl-2 after εV1-2 treatment and suggest that the expression of Bcl-2 is under the control of PKCε activity.

Numerous studies have implicated the increase of intracellular free Ca^{2+} in the initiation of apoptosis. Interventions resulting in elevations in cytosolic Ca^{2+} have been found to potentiate the phenomenon of apoptosis. Under our experimental conditions, control cardiocytes were quiescent and the addition of NaCN did not restore mechanical activity. Such a finding suggests that the increase of cytosolic Ca^{2+} with NaCN is mediated by an enhanced L-type calcium channel activity and mobilisation of calcium from intracellular stores. The saponin decreases the intracellular free Ca^{2+} concentration, and keeps the calcium balance (calcium overload could induce the apoptosis of cardiocytes). PKC inhibitor failed to increase the intracellular Ca^{2+} in cardiocytes (data not shown). However, it is not known how the PKC dependent pathway and the calcium dependent pathway interact with each other.

Taken together, the information provided by this investigation expands our understanding of the signalling mechanisms that NaCN could induce in cardiocyte apoptosis. The present study provides evidence that the saponin **1** can protect cardiocytes through decreasing the intracellular free Ca^{2+} concentration, increasing the Bcl-2 protein levels and activating the translocation of PKCε-mediated cellular signalling transduction pathways, which are associated with cardiocyte apoptosis.

3. Experimental

3.1 Materials

The saponin **1** was a gift from the Department of Chemistry, School of Basic Medical Sciences, Jilin University, its purity being over 98%, and it was dissolved in ethanol before use. NaCN, Fluo-3/AM, and antibodies against Bcl-2 were purchased from Newmarkers (USA), PKCε and phosph–PKCε rabbit polyclonal antibodies were obtained from Sigma (USA). Unless indicated, all other chemicals and materials were purchased from Sigma (USA).

3.2 Cell culture

Ventricular myocytes were isolated from the hearts of 1-day-old Sprague-Dawley rats by trypsinase digestion, as previously described [12]. Cardiocytes were preplated for 90 min in serum-free IMDM/medium to reduce non-myocyte contamination. The nonadherent cardiocytes were then plated at a density of 3×10^5 cells/ml on 60-mm culture dishes and 5×10^5 cells/ml in 24-well plates with IMDM containing 10% BSA; the culture medium was changed to IMDM with 10% BSA at 24 h after seeding. On culture day 3, the cardiocytes were then pretreated with the saponin or other agents for 30 min and subsequently stimulated with 3 mmol/L NaCN for the indicated times.

3.3 Evaluation of cell viability

Trypan blue exclusion was used to determine cardiocyte viability [13]. Isolated live cardiocytes were incubated with 0.4% Trypan blue dye for 3 min. Approximately 200 cells in

each of the eight dishes per experimental group were examined in a haemocytometer chamber under an inverted light microscope. Cells excluding the stain were considered viable and the percentage of non-blue cells was used as an index of viability.

3.4 Analysis for apoptosis rate

All the cells were collected, digested and washed twice in PBS, and the method was assayed as previously described [14]. The floating cells were precipitated with 0.2 ml PBS. After the cells were fixed in 70% precooled ethanol for 24 h, they were stained with PI and examined with flow cytometer.

3.5 Terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) assay

TUNEL assay was carried out as previously described [15]. Both cardiocytes floating in the media and trypsinised were collected together and used for the assay. TUNEL assay was performed on slides with a TACS 2 Tdt (TBL) kit (Trevigen). The number of TUNEL-positive cells was normalised to the total number of cells counted.

3.6 Ca^{2+} fluorescence image measured by laser scanning confocal microscopy

Cells (1×10^5/ml) cultured in sterile Petri dishes with coverslips for 12 h were loaded in IMDM containing 9 μM Fluo-3/AM and cultured at 37°C for 60 min in 5% CO_2 incubator. The labelled cells attached to coverslips were washed three times with PBS to remove the unloaded Fluo-3/AM in the medium. After loading with the fluorescence probes, the images of Ca^{2+} concentration in arbitrary single cells were monitored by Laser Confocal Scanning Microscopy (LSCM Leica, TCS-NT type, Germany) at 488 nm excitation wavelength.

3.7 PKC fluorescence image measured by laser scanning confocal microscopy

Cells were plated on glass coverslips, fixed for 10 min in PBS (pH 7.4) containing 4% paraformaldehyde and subsequently incubated for 10 min with 50 mM NH_4Cl. Fixed cells were permeabilised with 0.2% Triton X-100 in PBS-containing 2% BSA for 1–2 h at room temperature. To detect PKCε in cardiocytes, fixed cells were incubated with anti-PKCε antibodies (1:500) overnight at 4°C. Thereafter, cells were washed with PBS and followed by FITC IgG for 30 min at 37°C. The coverslips were mounted in fluorescent mounting medium and stored in the dark, and analysed by Laser Confocal Scanning Microscopy at 525 nm excitation wavelength.

3.8 Immunoblot analysis

Immunoblots were performed using an anti-Bcl-2, anti-phosphorylated PKCε. The films were developed using a Supersignal chemi-luminescence kit (Pierce). The photodensity of each band was quantitated using the Gel-Doc system.

3.9 Immunoblot analysis and translocation of PKC

Cells were harvested in the presence of lysis buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM EGTA, 5 mM EDTA, 20 μM leupeptin, 2 μg/ml aprotinin, 200 μM PMSF, and 5 mM

DTT, and sonicated. Protein concentration was determined with Bio-Rad protein assay kit (Bio-Rad), and 3 mg of each cardiocyte preparation was subject to differential centrifugation to collect the cytosolic and particulate fraction as described [15,16]. The percentages of individual PKCε and phosph–PKCε in each fraction were calculated as previously reported [15,16].

3.10 Immune complex kinase assay of PKC activity

Immune complex kinase assay of PKCε was performed as previously reported [15,17]. PKCε was immunoprecipitated using an anti-PKCε antibody from 2 mg whole-cell lysate. The kinase assay was carried out according to the methods described previously. The reaction mixture did not contain additional calcium acetate for this assay. The presence of PKCε was confirmed by immunoblot. PKCε activity was normalised for the amount of protein used and expressed as the PKCε activity measured relative to cardiocytes.

3.11 Statistical analysis

Data were expressed as means ± SD. To facilitate comparisons, measurements in each experiment were expressed as a percentage of the average value for the control group. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were performed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significantly different.

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