

Picroside II protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by activating the PI3K/Akt and CREB pathways

FAN-JI MENG, SU-MIN JIAO and BO YU

Department of Cardiology, The First Affiliated Hospital of China Medical University, Shenyang, P.R. China

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Abstract. Picroside II, an iridoid glucoside found in the root of *Picrorhiza scrophulariiflora* Pennell (Scrophulariaceae), has been demonstrated to reduce apoptosis in neuronal cells and other cell types. However, whether picroside II has a protective effect against cardiomyocyte apoptosis is poorly understood. In the present study, we investigated the cardioprotective role of picroside II and the underlying mechanisms in hypoxia/reoxygenation-induced cardiomyocyte apoptosis. The pretreatment with picroside II markedly attenuated hypoxia/reoxygenation-induced cell damage dose-dependently, which was evident by the increased cell viability and the corresponding decrease in lactate dehydrogenase release (LDH). The pretreatment with picroside II inhibited apoptosis confirmed by Annexin V-FITC staining, Hoechst 33258 nuclear staining and by assessment of caspase-3 activity. In addition, we found that picroside II not only increased the expression of Bcl-2, while decreasing Bax expression, but also augmented Akt and cAMP response element-binding protein (CREB) phosphorylation and ultimately inhibited hypoxia/reoxygenation-induced apoptosis. Furthermore, the protective effects of picroside II were abrogated by pretreatment of the cells with wortmannin or LY294002, a specific PI3K inhibitor. The present study suggests that picroside II inhibits hypoxia/reoxygenation-induced apoptosis in cardiomyocytes by activating the PI3K/Akt and CREB pathways and modulating expression of Bcl-2 and Bax.

Introduction

Apoptosis, a genetically programmed form of cell death, is a tightly regulated process to maintain tissue homeostasis and eliminate unwanted or dysfunctional cells in multicellular organ-

isms. The dysregulation of apoptosis can lead to a variety of heart diseases including heart failure and myocardial infarction with or without reperfusion injury (1,2). The role of apoptosis in heart diseases is becoming increasingly clear. Therefore, it is vital to develop a potential therapeutic strategy to block or prevent inappropriate apoptosis in heart diseases.

Picroside II (h-D-glucopyranoside, 1a, 1b, 2, 5a, 6, 6a-hexahydro-6-[(4-hydroxy-3-methoxybenzoyl)oxy]-1a(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-c]pyran-2-yl) is a major iridoid glucoside isolated from *Picrorhiza scrophulariiflora* Pennell (Scrophulariaceae) (Fig. 1) (3). Previous studies have shown that picroside II has a wide range of pharmacological effects, including neuroprotective (4,5), hepatoprotective (6), anti-oxidation (7), anticholestatic, anti-inflammatory and immunomodulating activities (8,9). In addition, picroside II is also reported to possess potential anti-apoptotic activities in hepatocytes (6), and rat model of focal cerebral ischemia (5). Therefore, it seems reasonable to investigate whether picroside II is able to protect cardiomyocytes against apoptosis.

Akt, a serine/threonine kinase, is the primary mediator of Phosphoinositide 3-kinase (PI3K)-initiated signaling. The PI3K/Akt pathway regulates the process of cell survival through phosphorylation of a variety of downstream targets such as the Bcl-2 family member Bad, caspase-9 and CREB (10). The pro- and anti-apoptotic members of the Bcl-2 family are intrinsic to the apoptotic pathway; Bcl-2 and Bcl-xL induce cell survival, whereas Bax and Bad promote cell death (11,12). It is widely accepted that Bcl-2 family, located in the outer membranes of the mitochondria, can regulate mitochondrial outer membrane permeability and trigger apoptosis (13). The Bcl-2 protein inhibits apoptosis by preventing the release of cytochrome c and the subsequent activation of caspases (14).

In the present study, we investigated the effect of picroside II on hypoxia/reoxygenation-induced apoptosis in cardiomyocytes and, most importantly, explored the possible underlying mechanisms.

Materials and methods

Animals. The animal use in this study was performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). The use of animals was

Correspondence to: Professor Bo Yu, Department of Cardiology, The First Affiliated Hospital of China Medical University, Liaoning, Shenyang 110001, P.R. China
E-mail: fanjimeng2060@yahoo.cn

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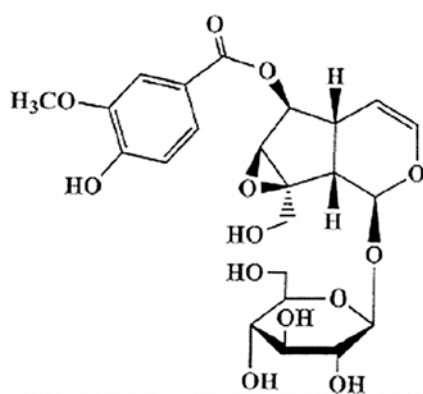


Figure 1. Chemical structure of picroside II.

also reviewed and approved by the China Medical University Animal Care Review Committee. Neonatal Sprague-Dawley 1- to 3-day-old rats [Grade II, Certificate Number SCXK-(Liao) 2009-0001] were purchased from China Medical University (Shenyang, China).

Chemicals and reagents. Picroside II (purity >99%), was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), DMEM glucose-free, new-born calf serum and trypsin were purchased from Gibco-BRL (Carlsbad, CA, USA). Collagenase II was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). ECL reagent kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Antibodies against Akt, phospho-Akt (Ser-473), CREB and phospho-CREB, were obtained from Bioworld Technology, Inc. (Minneapolis, MN, USA). anti-Bcl-2 antibody, anti-Bax antibody and anti- β -actin antibody was purchased from Boster Biological Technology, Ltd. (Wuhan, China). The detection kits of lactate dehydrogenase (LDH), were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). bicinchoninic acid (BCA) protein assay kit, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002) and wortmannin were supplied by Byeotime (Jianshu, China). Annexin V-FITC apoptosis detection kits were obtained from Nanjing KeyGen Biotech, Co, Ltd. (Nanjing, China). All other chemicals were purchased from Sigma-Aldrich. The purity of all reagents was at least analytical grade.

Primary culture of neonatal rat cardiomyocytes. The primary culture of neonatal rat cardiac ventricular myocytes was prepared from Sprague-Dawley rats (1- to 3-day-old) based on a previously published protocol with some modifications (15). Briefly, hearts were harvested and placed in ice-cold D-Hank's buffer. Ventricles were separated and cut into 1-mm³ pieces. The tissue fragments were digested by treatment with 0.06% trypsin once (37°C) and subsequently with 0.08% collagenase II 4-5 times (37°C). The cell suspension was then filtered and centrifuged for 8 min (120 x g, 4°C), and finally resuspended in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml). Resuspended cells were plated onto 6-well plates in a humidified incubator (5% CO₂, 95%

air, 37°C) for 2 h to exclude fibroblasts cells based on the fact that the fibroblasts cells attach to the surface more rapidly. To prevent proliferation of fibroblasts, 0.1 mmol/l 5-bromo-2'-deoxyuridine (BrdU) was added to the culture medium. Non-adherent cells were then replated onto fresh 96- and 6-well plates (1.2x10⁵ and 2x10⁶ cells/well, respectively) and incubated for 3-4 days before the experiment.

Hypoxia-reoxygenation model. In the present study, we used the model of hypoxia-reoxygenation *in vitro* which was similar to that described by Zhu *et al* (16). Briefly, before starting the experiments, the cultured cardiomyocytes were carefully washed 3 times with Hank's solution (5 mM HEPES, 137 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂; pH 7.2). The cells were incubated in a glucose-free DMEM base medium and then subjected to hypoxia to mimic the *in vivo* condition of myocardial ischemia. The cells were placed in an incubator at 37°C. N₂ (95%) and CO₂ (5%) were flushed into the incubator to bring the oxygen content down to 1% monitored by an oxygen probe. After 3 h of hypoxia, the cells were reoxygenated by immediately replacing a glucose-free DMEM base medium with a DMEM base medium with 5.5 mM glucose (pH 7.4) followed by incubation under normoxia for 2 h.

Experimental groups and protocols. After cardiomyocytes had been cultured for ~72 h, they were in the state of confluence and beat synchronously. The cultured cardiomyocytes were randomly divided into 7 groups: i) control group: cardiomyocytes were incubated under normoxic conditions at 37°C during the entire experimental period; ii) hypoxia/reoxygenation group: cardiomyocytes were incubated with a glucose-free DMEM base medium for 3 h of hypoxia followed by reoxygenation for 2 h as described above; iii) picroside II-50 group: picroside II (50 μ g/ml) was applied 48 h prior to hypoxia/reoxygenation; iv) picroside II-100 group: picroside II (100 μ g/ml) was applied 48 h prior to hypoxia/reoxygenation; v) picroside II (200 μ g/ml) group: picroside II (200 μ g/ml) was applied 48 h prior to hypoxia/reoxygenation; vi-vii) cells co-treated with hypoxia/reoxygenation and picroside II (200 μ g/ml) were challenged with the alternative PI3K inhibitor wortmannin (wortmannin, 50 nM: picroside II + wortmannin group) or 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, 15 μ M: picroside II + LY294002 group).

Cell viability assays. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cardiomyocytes were seeded on 96-well plates at 1.2x10⁵ cells/well. Following exposure to hypoxia-reoxygenation, cardiomyocytes were treated by the addition of 20 μ l MTT solution (5 mg/ml phosphate buffer) for 4 h and the media were removed. The formazan blue crystals, formed by oxidation of the MTT dye, were dissolved in 150 μ l DMSO for 10 min at the condition of vibration. The absorbance at 490 nm was measured using a microplate reader (Sunrise RC; Tecan Group, Ltd., Mannedorf, Switzerland) and cell viability was expressed as a percentage of the control culture value.

LDH activity assay. LDH activity was measured as the LDH content was released in the culture medium. After hypoxia/

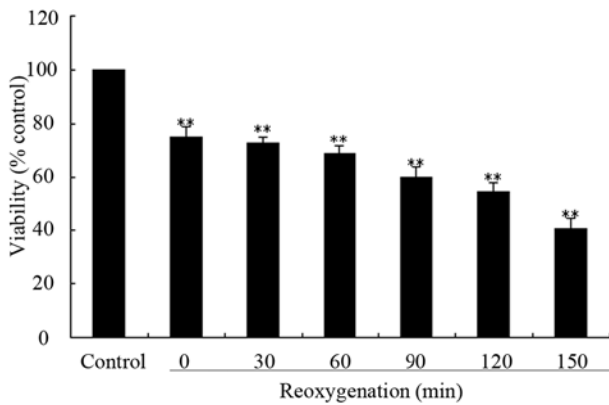


Figure 2. Time-dependent toxic effect of hypoxia/reoxygenation on cardiomyocyte viability. Cardiomyocytes were exposed to hypoxia 3 h/reoxygenation for 30-150 min. Viable cells were identified with MTT assay; data are shown as the mean \pm SD of 3 experiments; n=6; **P<0.01 vs. control.

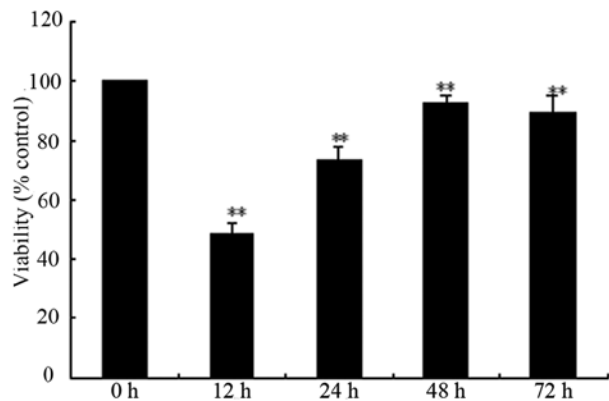


Figure 3. Cardiomyocytes were treated with 200 μ g/ml of picoside II for the times indicated. Values are means \pm SD from 5 independent experiments. **P<0.01 vs. the control group.

reoxygenation, 0.2 ml of culture medium was taken and assayed for LDH activity using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with a microplate reader (Sunrise RC; Tecan Group, Ltd.), following the manufacturer's instructions.

Caspase-3 activity assay. Caspase-3 activity was measured using the caspase-3 assay kit/colorimetric according to the manufacturer's instructions. Briefly, at the end of hypoxia/reoxygenation, 2×10^6 cells were collected, centrifuged $1,000 \times g$ for 10 min at 4°C . The cells were resuspended in the lysis buffer, incubated for 5 min on ice bath and centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant was then reacted with Ac-DEVD-pNA (2 mM), which were the substrates of caspase-3, at 37°C for 2 h. The protein concentration in the supernatant was determined by the BCA method. The absorbance at 405 nm was measured using a microplate reader (Sunrise RC; Tecan Group, Ltd.).

Nuclear staining for assessment of apoptosis. The nuclei of cardiomyocytes were stained with the chromatin dye Hoechst 33258. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After three washes with PBS,

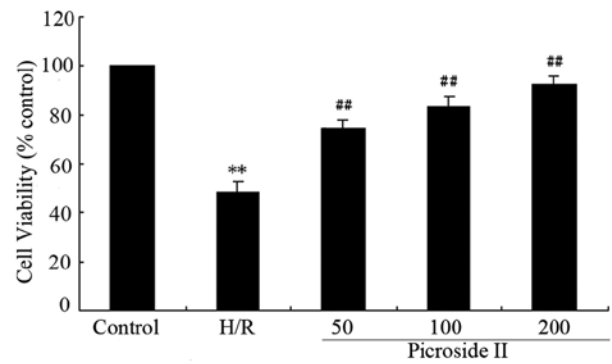


Figure 4. Effects of picoside II (50, 100 or 200 μ g/ml) on cell viability in cardiomyocytes subjected to H/R. Values are expressed as mean \pm SEM; n=8; **P<0.01 vs. the control group; **P<0.01 vs. the H/R group.

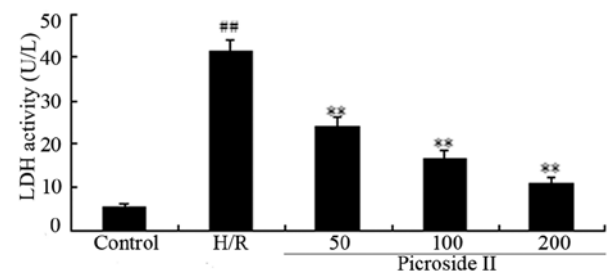


Figure 5. Effects of picoside II (50, 100 or 200 μ g/ml) on LDH activity in cardiomyocytes subjected to H/R. Values are expressed as mean \pm SEM; n=8; **P<0.01 vs. the control group; **P<0.01 vs. the H/R group.

the cells were incubated with Hoechst 33258 (5 μ g/ml in PBS) at room temperature for 15 min. After rinsing with PBS again, the cells were examined under a fluorescence microscope.

Analysis of flow cytometry. The apoptotic cells were measured by Annexin V-FITC/PI staining. At the end of hypoxia/reoxygenation, cells were harvested, washed with PBS, resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) and incubated with Annexin-V at room temperature in dark for 15 min. Then the cells were centrifuged, resuspended in the binding buffer and incubated with Propidium iodide (PI). After incubation, 400 μ l of binding buffer was added and the cells were analyzed by flow cytometry (FACScalibur; BD Biosciences, USA).

Western blot analysis. Cardiomyocytes with various treatments were lysed in an ice-cold RIPA buffer [1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mmol/l EDTA, 20 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 10 mmol/l NaF and 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF)]. The lysates were centrifuged at 12,000 rpm for 15 min at 4°C to remove debris. The protein concentration was determined with BCA protein assay. Equal amounts of denatured protein samples (50-100 μ g protein/lane) were separated by 10-12% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then washed with TTBS 3 times and incubated further with horseradish peroxidase-conjugated secondary antibody (1:2,000) for 2 h at room temperature.

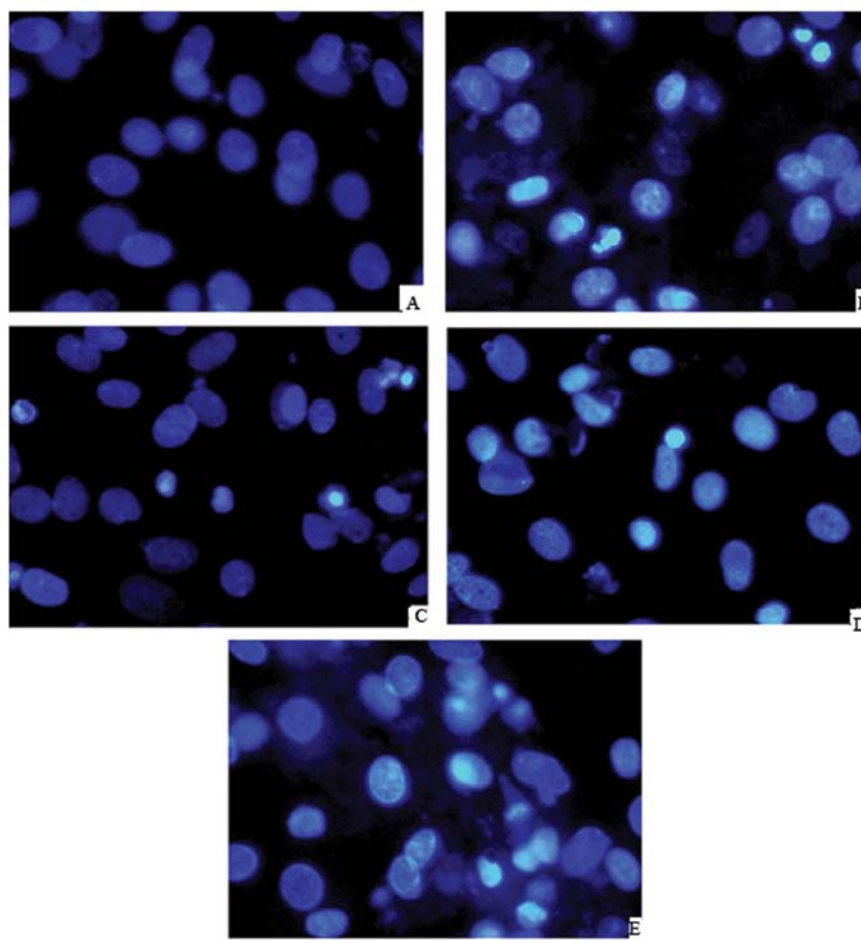


Figure 6. Effect of picroside II on histochemical characterization of hypoxia-induced apoptotic myocytes (magnification, x400). (A) Control; (B) hypoxia/reoxygenation; (C) hypoxia/reoxygenation + picroside II (200 $\mu\text{g/ml}$) group; (D) hypoxia/reoxygenation + picroside II (200 $\mu\text{g/ml}$) + LY294002 (10 μM) group (co-pretreatment of cells with LY294002 for 1 h); (E) hypoxia/reoxygenation + picroside II (200 $\mu\text{g/ml}$) + wortmannin (50 nM) group (co-pretreatment of cells with wortmannin for 1 h).

The blots were processed using an electrochemiluminescence (ECL) kit and light emission was captured on X-ray film. The blots were visualized with ECL-plus reagent and then subjected to densitometric analysis. β -actin was used as the internal loading control.

Statistical analysis. The data are expressed as the mean \pm SD. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Differences were considered to be statistically significant at $P < 0.05$.

Results

Effect of picroside II on the viability of cardiomyocytes subjected to hypoxia/reoxygenation. The results show that pre-incubation of cardiomyocytes with different concentrations of picroside II (0, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 $\mu\text{g/ml}$) prevented the loss of viability that resulted from hypoxia/reoxygenation in a dose-dependent manner (data not shown). Maximum viability was apparent at a concentration of 200 $\mu\text{g/ml}$. However, higher concentrations (400 and 800 $\mu\text{g/ml}$) of picroside II did not cause any enhancement of this preventive effect. We analyzed the effective half-maximal concentration for protection (EC_{50}) was 50 $\mu\text{g/ml}$. Therefore,

we used concentrations of picroside II (50, 100 or 200 $\mu\text{g/ml}$) for our subsequent experiments. As shown in Fig. 2, hypoxia 3 h/reoxygenation for 30-150 min significantly decreased the percentage of survival cells in a time-dependent manner. Subjected to hypoxia 3 h/reoxygenation for 120 min, there were only $54.29 \pm 3.4\%$ viable cells as compared to the control cells. Thus, hypoxia 3 h/reoxygenation 120 min was used as a standard apoptosis induction in the subsequent experiments.

Fig. 3 shows the results of the time-response investigation during which cells were exposed to 200 $\mu\text{g/ml}$ picroside II for ≤ 72 h. The magnitude of cell survival peaked at 48 h, when cell viability was $92.3 \pm 3.02\%$. Fig. 4 shows that after exposure to hypoxia 3 h/reoxygenation for 2 h the survival rate of cardiomyocytes was $48.25 \pm 4.31\%$. Pre-incubation with picroside II (50, 100 and 200 $\mu\text{g/ml}$) prevented cardiomyocytes from hypoxia/reoxygenation-induced damage, and caused a dose-dependent attenuation in cell survival to 74.17 ± 3.51 , 83 ± 4.5 and $92.02 \pm 3.6\%$, respectively (all $P < 0.01$ vs. hypoxia/reoxygenation).

Effect of picroside II on the levels of LDH in cardiomyocytes subjected to hypoxia/reoxygenation. The release of LDH in the medium is widely known as an indicator of cardiomyocyte injury. As shown in Fig. 5, LDH concentrations in medium

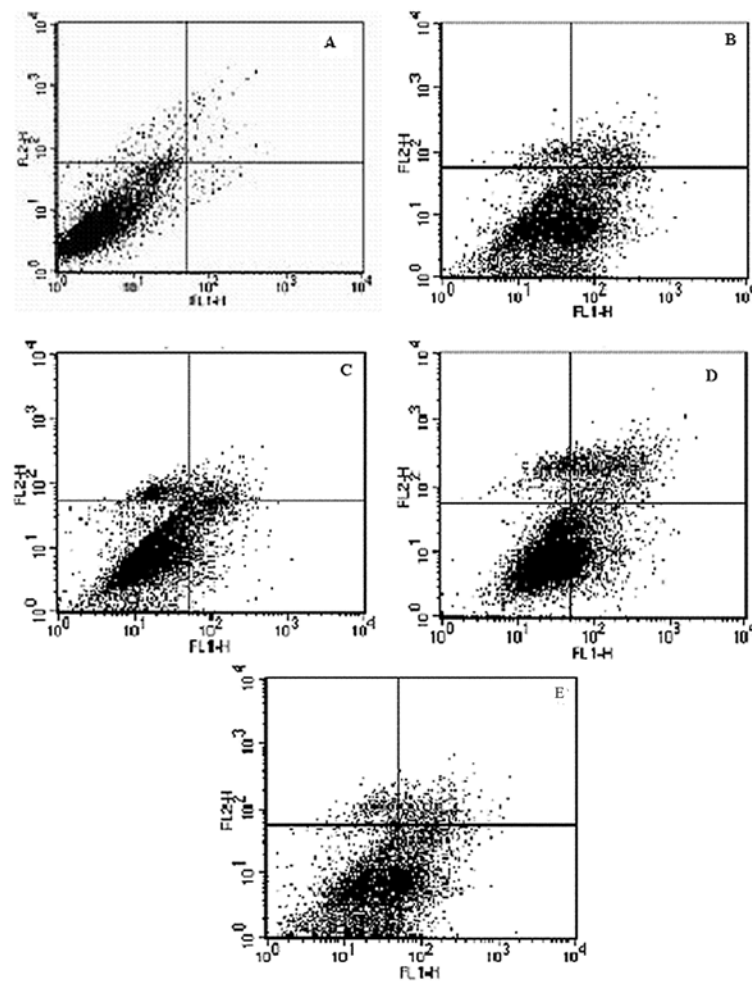


Figure 7. Effect of picoside II on hypoxia/reoxygenation-induced cardiomyocyte necrosis and apoptosis was determined with flow cytometry. (A) Control; (B) hypoxia/reoxygenation; (C) hypoxia/reoxygenation + picoside II (200 µg/ml) group; (D) hypoxia/reoxygenation + picoside II (200 µg/ml) + LY294002 (10 µM) group; (E) hypoxia/reoxygenation + picoside II (200 µg/ml) + wortmannin (50 nM) group.

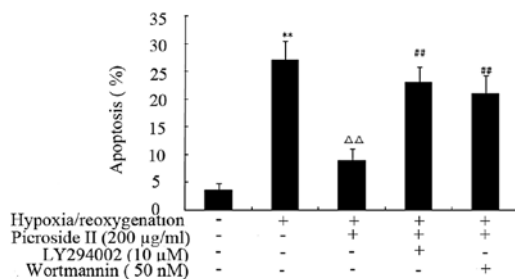


Figure 8. The percentage of apoptosis in cardiomyocytes from the control or the pretreated groups. All data were expressed as mean \pm SD; n=6; **P<0.01 vs. control, $\Delta\Delta$ P<0.01 vs. hypoxia/reoxygenation; ##P<0.01 vs. the hypoxia/reoxygenation + picoside II group.

from control group cells were minimal (5.63 ± 0.39 U/l), while LDH activity induced by hypoxia/reoxygenation increased up to (41.5 ± 2.71 U/l). Treatment with picoside II (50, 100 and 200 µg/ml) significantly attenuated LDH activity in a dose-dependent manner (concentrations were 24.02 ± 2.0 U/l, 16.69 ± 1.92 U/l and 10.09 ± 1.38 U/l, respectively).

Effect of picoside II on apoptosis in cardiomyocytes subjected to hypoxia/reoxygenation. Hoechst 33258 staining assay

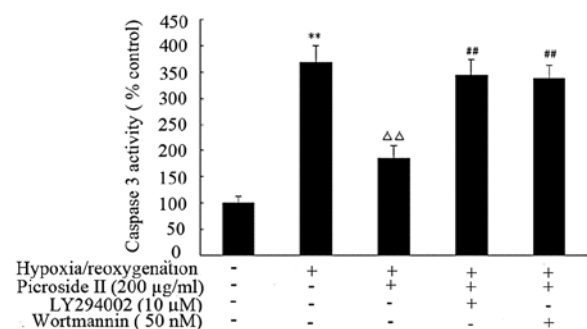


Figure 9. Inhibitory effect of picoside II on hypoxia/reoxygenation induced caspase-3 activity. Cells were pretreated with 200 µg/ml picoside II for 48 h and then exposed to hypoxia 3 h/reoxygenation 2 h. Protection of picoside II was abrogated by the PI3K inhibitor LY294002 or wortmannin treatment. Caspase-3 activity was determined as described in the Materials and methods (n=6). Data are presented as the mean \pm SD; n=6; **P<0.01 vs. control, $\Delta\Delta$ P<0.01 vs. hypoxia/reoxygenation; ##P<0.01 vs. the hypoxia/reoxygenation + picoside II group.

showed that cardiomyocytes exposed to hypoxia/reoxygenation featured typical characteristics of apoptosis, including the condensed chromatin and the fragmented apoptotic nuclei. However, the development of these apoptotic features were

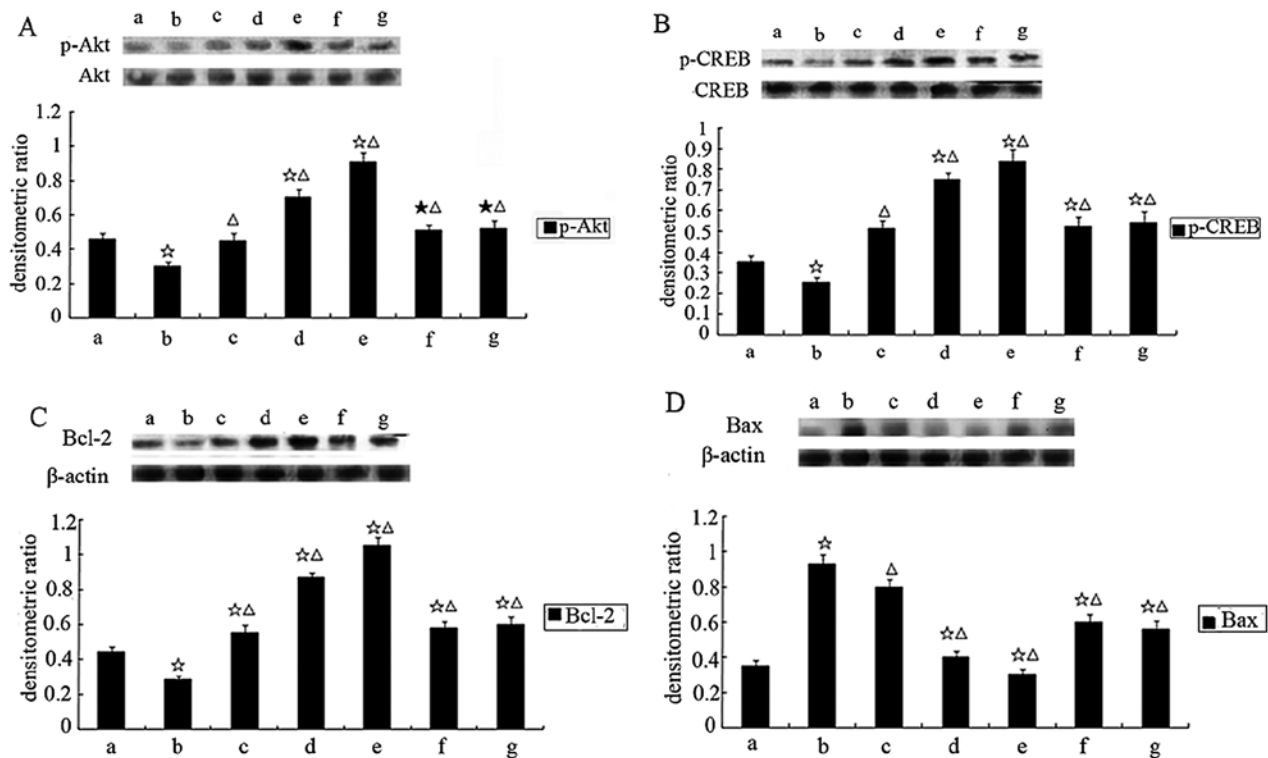


Figure 10. Representative western blot analysis showing the protein expression. (A) Phosphorylated Akt and total Akt; (B) phosphorylated CREB and total CREB; (C) Bcl-2; (D) Bax. a, control group; b, hypoxia/reoxygenation; c, 50 $\mu\text{g/ml}$ picroside II group; d, 100 $\mu\text{g/ml}$ picroside II group; e, 200 $\mu\text{g/ml}$ picroside II group; f, picroside II + LY294002 group; g, picroside II + wortmannin group. Data were expressed as densitometric ratio. Values are mean \pm SD; $n=3$; * $P<0.05$ and $\Delta P<0.01$ vs. the control group; $\Delta P<0.01$ vs. hypoxia/reoxygenation.

significantly suppressed when cells were treated with picroside II (200 $\mu\text{g/ml}$) (Fig. 6).

Flow cytometry revealed that hypoxia-reoxygenation caused a significant increase in the percentage of apoptotic cells from $3.3\pm0.95\%$ in control cells to $27.09\pm3.42\%$ ($P<0.01$) (Fig. 7). However, when picroside II (200 $\mu\text{g/ml}$) was applied 48 h prior to hypoxia-reoxygenation, it caused a reduction in the percentage of apoptotic cells to $9.05\pm2.0\%$ ($P<0.01$) (Fig. 8).

Cardiomyocytes exposed to hypoxia/reoxygenation insults had an increase in caspase-3 activation (368.3 ± 32.12) comparing to the control group (100 ± 12.01). Treatment with picroside II (200 $\mu\text{g/ml}$) for 48 h significantly decreased this hypoxia/reoxygenation-induced caspase-3 activation (Fig. 9).

However, the protection of picroside II was partially blocked by pretreating cells with wortmannin or LY294002. These results provide direct evidence that picroside II can significantly inhibit apoptosis in cardiomyocytes induced by hypoxia/reoxygenation, and that this effect is partially inhibited by concurrent wortmannin or LY294002 treatment.

Effect of picroside II on the phosphorylation of Akt and CREB in cardiomyocytes exposed to hypoxia/reoxygenation.

The kinetics of Akt activation were examined, to evaluate a possible mechanism for the anti-apoptotic effect of picroside II. As shown in Fig. 10A, hypoxia/reoxygenation significantly reduced the phosphorylation of Akt without alteration of the total Akt expression. Pretreatment of cardiomyocytes with picroside II, prior to hypoxia/reoxygenation exposure, resulted in a significant increase in the phosphorylation of Akt. In addition, CREB, a downstream target of Akt, was also

markedly phosphorylated (Fig. 10B). Both 50 nM wortmannin and 15 μM LY294002 markedly inhibited picroside II-induced Akt and CREB phosphorylation.

Effect of picroside II on the phosphorylation of Bcl-2 and Bax in cardiomyocytes exposed to hypoxia/reoxygenation. Akt phosphorylates the transcription factor CREB, implicated in the transcription of the anti-apoptotic Bcl-2 gene (17). We investigated the protein expression of the anti-apoptotic Bcl-2 and the pro-apoptotic Bax. As shown in Fig. 10C, the expression of Bcl-2 protein decreased in the hypoxia/reoxygenation group compared to the control group. However, cells treated with picroside II showed an increase in Bcl-2 protein expression as detected by western blot analysis. Picroside II pretreatment downregulated Bax levels in cells exposed to hypoxia/reoxygenation comparing to untreated cells (Fig. 10D). This effect was partially inhibited by concurrent wortmannin or LY294002 treatment. Picroside II treatment mediated phosphorylation of Akt and CREB, increased Bcl-2 protein expression, and decreased Bax protein expression and caspase-3 activity in cardiomyocytes hypoxia/reoxygenation, which was abolished by LY294002 or wortmannin treatment.

Discussion

Through examination, we firstly proved the cardioprotective effects of picroside II on apoptosis induced by hypoxia/reoxygenation in a neonatal rat cardiomyocyte. In the present study, we demonstrated that pretreatment of cardiomyocytes with picroside II (50–200 $\mu\text{g/ml}$) for 48 h significantly reduced

the decrease in viability and LDH activities associated with exposure to hypoxia/reoxygenation as determined by MTT and LDH assays. Picroside II decreased hypoxia/reoxygenation-induced apoptosis in cardiomyocytes as indicated by nuclear morphological Hoechst staining, flow cytometry and caspase-3 activity. These results indicate that picroside II protects against hypoxia/reoxygenation-induced apoptosis in that it not only involves enhanced phosphorylation of Akt and CREB, but also upregulation of Bcl-2 and downregulation of Bax via the PI3-kinase/Akt signaling pathway.

Picroside II is a major iridoid glucoside isolated from *Picrorhiza scrophulariiflora* Pennell (Scrophulariaceae) (3). Previous studies have shown that picroside II has a number of pharmacological effects, including neuroprotective (4,5), hepatoprotective (6), antioxidant, anticholestatic, anti-inflammatory and immunomodulating activities (7,8). In addition, picroside II is also reported to possess potent anti-apoptotic activities (5,6). It has been reported to stimulate anti-apoptotic proteins such as Bcl-2 (6) and inhibit proteins with pro-apoptotic properties such as caspase-3 and poly ADP-ribose polymerase (PARP) (5). The most demonstrated findings are from studies in neuronal cells (4,5). In the present study, we examined the effect of picroside II on the viability of cardiomyocytes subjected to hypoxia/reoxygenation by MTT and LDH assays. Apoptosis was also assessed by Hoechst 33258 staining, Annexin V-FITC staining and caspase-3 activity. We found that picroside II protected cardiomyocytes from hypoxia/reoxygenation-induced decrease in cell viability and apoptosis.

The mechanism by which picroside II protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis is an important issue raised by the results presented in this study. Multiple signaling pathways participate in regulation of cell survival. Our study emphasized on examining potential changes in the Akt-CREB pathway. Akt is a critical regulator of PI3-kinase-mediated cell proliferation and survival (18). Akt is involved in many cell anabolic processes, including glucose transport, glycogen synthesis, and protein synthesis (19). Akt can also regulate the process of cell survival through phosphorylation of diverse target molecules including the Bcl-2-family member Bad, Forkhead, caspase-9, NF- κ B and CREB (20,21).

Akt also activates cAMP response element binding (CREB) protein, stimulates recruitment of CBP to the promoter and promotes cellular survival through a CRE-dependent mechanism (22,23). The transcription factor CREB mediates survival in many cells, including cardiomyocytes. It has been shown that Bcl-2 is one of the most important CREB-regulated genes, playing a critical role in the cell survival (24). The promoter region of Bcl-2 contains a cAMP-response element site and CREB is known as a positive regulator of Bcl-2 expression (25-27). Furthermore, activated CREB can also inhibit caspase-3 activation and finally attenuate myocyte apoptosis (28). In line with this notion, our results showed that picroside II upregulated Bcl-2 protein expression and inhibited caspase-3 activation in the hypoxia/reoxygenation myocytes, which was accompanied by a significant increase of CREB phosphorylation. In this study, we found that Akt predominantly existed in an inactivated form in cardiomyocytes that were not undergoing hypoxia/reoxygenation, but was activated and phosphorylated when cells were co-treated with hypoxia/reoxygenation and picroside II. Picroside II markedly increased the phosphory-

lation of Akt and CREB. This activation was significantly blocked by the PI3K inhibitor wortmannin and LY294002. These results suggest that picroside II may protect cardiomyocytes from hypoxia/reoxygenation-induced apoptosis at least in part by activating the PI3K/Akt-CREB signaling pathway.

Many genes have been reported to be involved in the regulation of apoptosis, in which Bcl-2 and Bax genes are suggested to play a major role in maintaining the balance of cell death and survival (29,30). The main site of action of Bcl-2 family proteins appears to be the mitochondrion. Several studies have shown that the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax of the Bcl-2 family are associated with the mitochondrial membrane and affect membrane permeability (31). Bcl-2 prevent the release of cytochrome c from the mitochondria by binding to the pro-apoptotic proteins Bad, Bax and Bak (32,33). Thus, it can inhibit activation of caspases, such as caspase-9 and caspase-3, and prevent apoptosis (34,35). Moreover, it has been shown that upregulation of Bcl-2 can antagonize the pro-apoptotic activities of Bax and Bak (36). To further support our findings, the effects of picroside II on expression of Bcl-2 and Bax proteins were detected by western blot analysis. In the present study, we found that hypoxia/reoxygenation downregulated the expression of Bcl-2, whereas upregulated Bax expression. Pretreatment with picroside II ameliorated these changes in Bax and Bcl-2 protein expression. Furthermore, these effects were partially abolished by the PI3K inhibitor wortmannin and LY294002. Our findings suggest that Bcl-2 and Bax are involved in mediating the anti-apoptotic effects associated with picroside II treatment in cardiomyocytes exposed to hypoxia/reoxygenation.

In conclusion, our study for the first time demonstrates, that picroside II protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis. Our results also suggest that the anti-apoptotic effects associated with picroside II pretreatment are at least in part due to inhibited caspase-3 activation, activation of the PI3K/Akt/CREB signaling pathway and modulated Bcl-2 and Bax expression. Picroside II may hold promise as a therapeutic intervention for the treatment of myocardial ischemia/reperfusion. Further detailed investigation is needed in these fields.

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