Polydatin attenuates ischemia/reperfusion-induced apoptosis in myocardium of the rat

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Abstract: The aim of the present study was to investigate the effect of polydatin on apoptosis induced by ischemia/reperfusion (I/R) in rat myocardium and to explore the underlying mechanism. Adult male Sprague-Dawley (SD) rats were randomly divided into control, I/R and polydatin (50 μmol/L) groups. On the Langendorff apparatus, isolated rat heart was subjected to 30-min global ischemia followed by 60-min reperfusion. TUNEL labeling and flow cytometric techniques were used for the measurement of apoptosis and the expression of Bcl-2 and Bax protein in cardiomyocytes of rat. The results showed: (1) Compared with those in the control group, the number of TUNEL-positive cells and apoptosis rate were increased in I/R group; (2) Compared with that in the I/R group, the number of TUNEL-positive cells was significantly decreased in the polydatin group [(18.1±4.0)% vs (35.1±5.4)%, P<0.01]; (3) Apoptosis rate assayed by flow cytometry in I/R group was significantly higher than that in polydatin group [(15.43±4.55)% vs (8.66±3.18)%, P<0.01]; (4) Expression level of Bax protein was higher in I/R group than that in polydatin group (P<0.05), while the level of Bcl-2 protein and Bcl-2/Bax ratio were higher in polydatin group than those in I/R group (P<0.05, P<0.01), respectively. The results obtained suggest that polydatin exerts an inhibitory effect on I/R-induced apoptosis through increasing Bcl-2 protein expression and decreasing Bax protein expression in myocardium of the rat.

Key words: polydatin; apoptosis; ischemia/reperfusion; heart; rat
Polydatin, 3, 4', 5-trihydroxystibene-3-β-mono-D-glucoside is a stilbene compound isolated from the dried roots of Polygonum Cuspidatum Sieb. et Zucc. Experimental studies showed that polydatin exhibited a lot of pharmacological activities, such as inhibiting the platelet aggregation, improving microcirculation, increasing survival rate of shocked rats and suppressing lipid peroxidation[1,3]. Our previous studies showed that polydatin had a cardioprotective effect against ischemic/reperfusion (I/R) injury. The proposed mechanisms for cardioprotection of polydatin include the increase of antioxidant enzymes activity, amelioration of coronary circulation, and involvement of NO[4]. Recently, we found that the cardioprotection of polydatin was related with the opening of ATP-sensitive potassium channel in both cell membrane and mitochondrial membrane, and the inhibition of mitochondrial permeability transition pore opening[5]. However, the precise mechanisms underlying the protective effects of polydatin on ischemic hearts are far from clear.

It has been proved that cardiomyocyte apoptosis played a significant role in myocardial damage induced by I/R[6,7]. Apoptosis, a form of death characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and genomic DNA fragmentation, is essential for development and maintenance of tissue homeostasis. On the other hand, apoptosis has been implicated in many diseases such as congestive heart failure and ischemic injury. It is believed that cell loss during cardiac I/R results from necrosis. There are increasing lines of evidence that the death of cardiac myocyte also results from apoptosis[7,8]. Bcl-2 family of proteins is proved to play a major role in determining the sensitivity or resistance of cells to a great number of stimuli that can induce apoptosis[9,10]. It is not known, however, whether the cardioprotective effect of polydatin relates to apoptosis during I/R. The objective of this study was to investigate the effect of polydatin on I/R-induced apoptosis in rat heart and the underlying mechanism.

1 MATERIALS AND METHODS

1.1 Experimental materials
Adult male Sprague-Dawley (SD) rats weighing 280-320 g. (grade II, Certificate No. 04036), were provided by the Experimental Animal Center of Hebei Province. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Reasearch Council, 1996).

Polydatin was provided by Neptunus Pharmaceutical Co. Ltd (Shenzhen, China). The antibodies for immunofluorescence detection of Bcl-2 and Bax protein were as follow: Monoclonal antibodies of Bcl-2 (C-2, mouse against human protein, working concentration 1:100) and Bax (B-9, mouse against mouse protein, working concentration 1: 100) were purchased from Santa Cruz Biotechnology Inc., USA. The second antibody, FITC-conjugated goat anti-mouse IgG, was provided by Jackson ImmunoResearch Laboratories Inc., USA.

1.2 In vitro heart experiment
Rats were anesthetized with sodium pentobarbital (30 mg/ kg, i.p.) and hearts were quickly excised and mounted on a Langendorff apparatus via aorta for retrograde perfusion with Krebs-Henseleit (K-H) buffer at constant pressure (100 cm H2O). K-H solution was composed of (in mmol/L): NaCl 118.0, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25.0, KH2PO4 1.2, glucose 11.0, gassed with 95% O2 and 5% CO2 (pH 7.4) and maintained at 37°C. A water-filled latex balloon connected to a pressure transducer (Gould P23Db) was introduced into the left ventricle through atria to record isovolumic pressure of left ventricle. The balloon volume was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mmHg during initial equilibration. In the experiments, rats were randomly divided into three groups: (1) control group: hearts were subjected to normal perfusion with K-H buffer solution for 120 min; (2) I/R group: after stabilization for 30 min with K-H buffer solution, the hearts were subjected to 30 min of no-flow global ischemia followed by 60 min of reperfusion; (3) Polydatin group: the hearts were pretreated with 50 µmol/L polydatin for 10 min before I/R treatment. 1.3 TdT-mediated dUTP in situ nick-end labeling (TUNEL) TUNEL method was used to evaluate apoptotic activity. At the end of the experiment, the hearts were sectioned and fixed up in neutral formalin (10% formaldehyde in PBS, pH 7.4). Each section was deparaffinized and rehydrated with serial changes of xylene and ethanol. Proteinase K (20 µg/mL) was applied to the section for 15 min with the intention of producing optimal proteolysis. A commercial apoptosis detection kit (In Situ Cell Apoptosis Detection Kit I, POD) was used. Endogenous peroxidase was inhibited by 3% hydrogen peroxide for 5 min. The TdT reaction was continued for 1 h at 37°C, and then antidigoxigenin-peroxidase was applied for 30 min at room temperature. Hematoxylin-Eosin was used as a counterstaining. For quantitative analysis, TUNEL-positive cells in five different slides from different hearts were counted. The data were expressed as the percentage of TUNEL-positive myocytes per slide.
1.4 Determination of apoptosis and Bcl-2 or Bax expression with flow cytometric measurements

Heart tissue was cut into small pieces and was rinsed with normal saline. Cardiac myocytes were collected and fixed in 70% ethanol at 4 °C overnight, and then cell suspension was collected. Cell suspension (1×10^6 cells/mL) were centrifuged (5 min, 1 000 r/min) and washed twice with 0.9% NaCl solution. After centrifugation the cells were stained in propidium iodide (PI) solution (PI 50 µg/mL containing Triton-X-100 and RNase) for 30 min and filtered through a 47 µm nylon mesh to remove cellular fragment and cluster. Chicken red blood cells were added to the sample before staining as an internal standard for calibration of the flow cytometry (FCM) instrument.

The sample fluorescence staining was performed using indirect immunofluorescence labeling method. Each sample (1×10^6 cells/mL) was washed twice with PBS and incubated with 100 µL antibody (Bcl-2 or Bax) for 30 min at 37 °C. The samples were then washed twice with PBS and incubated with 100 µL of the second antibody for 30 min at 37 °C. Then the cell suspensions were washed, resuspended in 1.0 mL PBS, filtered through 47 µm nylon mesh, analyzed by FCM, which was similar to that previously described by Ji et al.[11]. The stained samples were analyzed by FCM instrument (FACS 420 Fluorescence Activated Cell Sorting, Becton. Dickinson, Sunnyvale. California, USA). Fluorescence was excited at 488 nm by a 15 mW Ar+ laser promoter. Single parameter was measured respectively in DNA (with a liner mode) and each protein (with a Log mode). Usually, 1×10^4 cells for each sample were analyzed. The analytic data were processed with a HP-300 consort 300 computer. The coefficient of variation (CV) of the instrument was adjusted within 2% using PI staining of chicken red blood cell. Fluorescence index (FI, the ratio of average fluorescence intensity of sample to average fluorescence intensity of normal control) was used to describe Bcl-2 and Bax protein expression.

1.5 Statistical analysis

All data were expressed as mean±SD. The paired t-test was used to compare the data within groups and ANOVA was used to compare the data among groups. P<0.05 was considered statistically significant.

2 RESULTS

2.1 Effect of polydatin on apoptosis induced by I/R assayed by TUNEL

TUNEL-positive cells showed a typical apoptosis. The cell size was obviously reduced, cytoplasm was shranked but plasma membrane was integral. The nuclei were pyknosis and marginated to the periphery of cell membrane, which indicated the condensation of chromatin. Few TUNEL-positive cells were observed in continuously perfused hearts from control group. While in I/R group, TUNEL-positive cells were found more frequently (Fig. 1), but in the polydatin group, the number of TUNEL-positive cells was significantly decreased [(18.1±4.0)% vs (35.1±5.4)%, P< 0.01)]. These results demonstrated that apoptosis in the myocardium after I/R was attenuated by polydatin.

2.2 Effect of polydatin on apoptosis induced by I/R assayed by FCM

FCM was used to estimate the number of apoptotic cells through counting sub-G1 cells. The analysis of cellular DNA content showed that there was a sub-G0/G1 peak, which was a typical apoptotic peak, in the graph. The apoptosis rate in I/R group was significantly greater than that in polydatin group [(15.43±4.55)% vs (8.66±3.18)% , P< 0.01] (Fig. 2).

2.3 Effect of polydatin on Bcl-2 and Bax protein expression under I/R detected by FCM

Compared with control group, FI values of Bax protein of

![Fig. 1. Comparison of apoptosis level in control group (A), I/R group (B) and polydatin group (C). Apoptosis was assayed by TUNEL and cells in brown (as shown by arrows) are positive ones. Compared with that in I/R group, the number of positive cells was significantly decreased in the polydatin group [18.1±4.0)% vs (35.1±5.4)% , P< 0.01]. Scale bar, 50 μm.](image-url)
DISCUSSION

Our previous study showed that polydatin could protect heart against I/R injury\(^4\), which was related with the opening of ATP-sensitive potassium channel in both cell membrane and mitochondrial membrane, and inhibition of mitochondrial permeability transition pore opening\(^5\), but there was no report about the effect of polydatin on the apoptosis of cardiomyocytes. The present study demonstrated that polydatin significantly reduced the apoptosis induced by I/R in the isolated rat heart, which suggested that the antiapoptotic effect might be one of mechanism for the cardioprotection of polydatin.

Increasing lines of evidence suggest that lethal reperfusion injury possibly consists of two forms of cell death, necrosis and apoptosis (programmed cell death)\(^6\-8\). The apoptotic process is initiated shortly after the onset of ischemia, and becomes markedly enhanced during myocardium in I/R group increased significantly \(P<0.01\), while both FI values of Bcl-2 protein and Bcl-2/Bax ratio decreased significantly (all \(P<0.05\)). Polydatin can eliminate the inhibitory effect of I/R on Bcl-2 expression, for Bcl-2 expression and Bcl-2/Bax ratio were augmented in polydatin group \((P<0.05, P<0.01\) vs I/R group\) (Table 1).

Table 1. Changes of Bcl-2 and Bax protein expressions in ventricular myocardium detected by flow cytometry

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluorescence index (FI)</th>
<th>Bcl-2/Bax ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bcl-2</td>
<td>Bax</td>
</tr>
<tr>
<td>Control</td>
<td>1.00(\pm)0.05</td>
<td>1.00(\pm)0.01</td>
</tr>
<tr>
<td>I/R</td>
<td>0.92(\pm)0.14*</td>
<td>1.23(\pm)0.10**</td>
</tr>
<tr>
<td>Polydatin</td>
<td>1.12(\pm)0.07#</td>
<td>1.04(\pm)0.16##</td>
</tr>
</tbody>
</table>

Mean\(\pm\)SD, \(n=6\). \*\(P<0.05\), \**P<0.01\) vs control group; \#\(P<0.05\), \##\(P<0.01\) vs I/R group.

3 DISCUSSION

Our previous study showed that polydatin could protect heart against I/R injury\(^4\), which was related with the opening of ATP-sensitive potassium channel in both cell membrane and mitochondrial membrane, and inhibition of mitochondrial permeability transition pore opening\(^5\), but there was no report about the effect of polydatin on the apoptosis of cardiomyocytes. The present study demonstrated that polydatin significantly reduced the apoptosis induced by I/R in the isolated rat heart, which suggested that the antiapoptotic effect might be one of mechanism for the cardioprotection of polydatin.

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reperfusion. Inhibition of the apoptotic process should then attenuate the irreversible injury in connection with reperfusion. Ischemic preconditioning, a phenomenon whereby exposure of the hearts to brief periods of ischemia, enhances the resistance of hearts to subsequent lethal ischemic injury, and has been shown to inhibit apoptosis in the heart subjected to I/R\textsuperscript{12,13}. Similar to ischemic preconditioning, the results from TUNEL-positive nuclei count in the present study, showed that polydatin could effectively decrease the cardiac apoptosis induced by I/R, which suggested that anti-apoptosis might be a mechanism of cardiac protection of polydatin on heart against I/R injury.

Apoptosis is a procedure involving a number of regulatory genes mediated by apoptosis signals. Among those, the Bcl-2 family of proteins constitutes a central checkpoint, which play an important role as both cell death promoter and cell death preventer\textsuperscript{14}. Pro-apoptotic proteins include Bax, Bak, Bcl-XS, Bad, Bik, Bim, Bid, Hrk and Bok, whereas anti-apoptotic proteins include Bcl-2, Bcl-XL, Mcl-1 and A1/Bfl-1. The ratios of anti- to pro-apoptotic molecules, such as the ratio of Bcl-2/Bax, determine the response to a death signal. Bcl-2, a 26 kDa protein, is predominantly localized to the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope. Bcl-2 seems to prevent apoptosis induced by many stimuli and it has been shown to suppress cytochrome c (Cyt c) efflux from mitochondria, inhibit calcium release from the endoplasmic reticulum\textsuperscript{15,16}. Others have speculated that Bcl-2 regulates the generation of reactive oxygen radical since Bcl-2 is able to prevent cell death induced by oxidative damaging agents\textsuperscript{17}. The anti-apoptotic role of Bcl-2 is well documented in myocardium. It was demonstrated that genetic modification of the myocardium with the anti-apoptotic human Bcl-2 gene conferred myocardial protection against I/R-induced apoptosis\textsuperscript{18,19}. Bax, which normally resides in the cytosol, translocates to mitochondria when triggered by certain stimuli\textsuperscript{20}. Translocated Bax forms large number of channels in the outer membrane of mitochondria, which induces Cyt c release and is followed by caspase activation, while Bcl-2 may be able to prevent channel formation by Bax\textsuperscript{14,20}. Therefore the ratio of Bcl-2 to Bax determines the fate of myocardial cell following apoptotic stimulus. Several studies have reported a reduction of apoptosis by ischemic preconditioning via altering the expression of Bcl-2 and/or Bax protein, thus increasing the ratio of Bcl-2/Bax protein and preventing the progression of apoptosis in myocardium after I/R\textsuperscript{14,15}. In the present study, the expression of Bax in the polydatin group hearts was decreased and the expression of Bcl-2 was increased after I/R, which suggested that the increased ratio of Bcl-2/Bax induced by polydatin might play a pivotal role for the anti-apoptosis of polydatin.

In conclusion, the present study demonstrated that polydatin decreased the apoptosis through increasing the ratio of Bcl-2/Bax, which might be one of mechanisms for protective effect of polydatin on heart against I/R injury.

REFERENCES

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