Basic Investigations

Study of Anti-Myocardial Cell Oxidative Stress Action and Effect of Tanshinone IIA on Prohibitin Expression

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**Objective:** To investigate the protective action of tanshinone IIA (TSN) on myocardial apoptosis induced by hydrogen peroxide ($H_2O_2$) and its effect on prohibitin (PHB) expression to probe the role of PHB in the oxidation stress of myocardial cells.

**Methods:** Primary cultured neonate rat myocardial cells were cultured with TSN ($1\times10^{-4}$ mol/L) for 24 hours, and then the medium was supplemented with 200 mol/L hydrogen peroxide for 2 h to initiate myocardial cell oxidative stress injury. PHB in myocardial cells was knocked down by small interfering RNA (siRNA), and the expression level of PHB was determined by western blot analysis. Flow cytometry was used to detect the apoptosis rate, intracellular calcium ion concentration ([Ca$^{2+}$]i) and mitochondrial membrane potential (MMP).

**Results:** The PHB expression, [Ca$^{2+}$]i and the apoptotic rate significantly increased, and the MMP significantly decreased in the oxidative stress group compared with the control. The PHB expression, apoptosis rate and [Ca$^{2+}$]i decreased, and MMP increased significantly in the TSN group compared with the oxidative stress group. Compared with the siRNA negative control group, the PHB expression level in myocardial cells was down-regulated, and the apoptosis rate and [Ca$^{2+}$]i increased, and MMP decreased significantly in the siRNA group.

**Conclusion:** TSN can reduce PHB expression in oxidative stress-injured myocardial cells hence protecting the myocardial cells.

**Keywords:** tanshinone IIA; prohibitin (PHB); myocardial cell; apoptosis; oxidative stress; calcium overload

With the wide use of coronary thrombolysis, coronary saccular dilatation and coronary artery bypass grafting for treatment of ischemic heart diseases, ischemia-reperfusion injury (I/RI) is an emergent problem. Many experimental studies have shown that a large number of free radicals are produced in the injured myocardial area, and oxidative injury from the free radicals contributes to overall myocardial injury. Free radical scavengers have been shown to alleviate this injury.

Prohibitin (PHB) is located in the inner mitochondrial membrane and has been shown to inhibit tumor cell proliferation, regulate cell cycles and apoptosis, protect mitochondrial function, and prevent ageing. Also, PHB has anti-inflammatory and anti-apoptosis effects in intestinal tissues. Proteomics studies indicate that PHB expression increases in myocardial ischemia-reperfusion; however, there are few reports about its role in myocardial injury.

Danshen (丹参 Radix Salvia miltiorrhiza) is a well-known traditional Chinese herb. Tanshinone IIA (TSN) is an important active component of Danshen and has been widely used for the treatment of coronary heart disease. In the present experiment, the protective action of TSN on myocardial cells and its regulative effects on PHB expression under conditions of oxidative stress were investigated. The function of PHB was studied with small interfering RNA (siRNA) interference technique in injured myocardial cells induced by hydrogen peroxide ($H_2O_2$) in vitro to elucidate the molecular mechanism of Dansen for the treatment of myocardial ischemic diseases.

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This study is supported by a grant from the National Natural Sciences Foundation of China (No. 30572435)
MATERIALS AND METHODS

Animals
1–3 day-old SPF Wistar neonatal male rats were purchased from the Experimental Animal Center, Southern Medical University (Certificate of quality: SCXK 粤-20060015).

Medicines and Reagents
Trypsin and DMEM culture medium were purchased from Gibco (USA); siPORT NeoFX transfection kit was purchased from Ambion (USA); fetal calf serum was purchased from Hangzhou Sijiqing Biotech Institute; TSN was purchased from China Medicines and Biological Products Test Institute (Batch No. 110766-200417). Prohibitin antibody from the mouse, and goat anti-mouse IgG-HRP, prohibitin siRNA and transfection reagents were purchased from Santa Cruz Biotechnology (USA). ECL chemiluminescence kit and protein quantitative determination kit, Fluo-3/AM calcium ion fluorescence probe, rhodamine 123 were purchased from Biyuntian Biotech Institute, and AnnexinV-FITC cell apoptosis detection kit was purchased from Nanjing Key Gen Biotech Development Co.

Grouping
After primary cultured neonate rat myocardial cells were separated and purified, six experimental groups were identified: 1) normal control group-myocardial cells under normal growth conditions; 2) oxidative stress group-myocardial cells growing in the medium supplemented with 200 µmol/L H₂O₂ for 2 h; 3) TSN + oxidative stress group-myocardial cells growing in the medium supplemented with TSN (1×10⁻⁴ mol/L) for 24 h before growing under oxidative stress conditions for 2 h; 4) siRNA group-myocardial cells transfected with specific PHB small interfering RNA; 5) siRNA + oxidative stress group-after transfection with specific PHB small interfering RNA, myocardial cells were cultured in the medium supplemented with 200 µmol/L H₂O₂ for 2 h; 6) siRNA negative control group-the normal cultured myocardial cells were transfected with negative control siRNA.

Culture of Neonate Rat Myocardial Cells
Primary myocardial cells from neonatal rats were cultured according to the procedure described by Goldenberg et al. In brief, the hearts were surgically taken from 1- to 3-day-old Wistar rats immerged in 75% alcohol for 8 s, and the cardiac atrium and epicardial connective tissue were removed and the ventricle of heart was open and washed for three times with cold D-Hanks solution in a culture dish. The muscles of the ventricle of the heart were minced into 1–2 mm³ pieces by aseptic operation and put into a 5 ml-tube containing 0.1% trypsin solution at 37°C, for 6 min. After natural sedimentation, the first supernatant was dispensed, and the precipitate was added to 5 ml of trypsin at 37°C for 6 min and gently brown. After natural sedimentation, the supernatant was moved into a 15 ml-centrifuge tube containing medium with 5% fetal calf serum to end the trypsinization, and centrifuged at 1000 rpm for 10 min and washed twice. The precipitate was added to fresh trypsin to continue digestion. Repeat trypsinization was conducted 7–10 times until digestion of all the tissue pieces was complete. The final precipitate was used to prepare a cell suspension in the presence of medium containing 15% fetal calf serum and cultured in a CO₂ incubator for 1 h, and the non-myocardial cells including mainly fibroblast and endothelial cells were removed. One hour later, the cell suspension was adjusted for cell density and inoculated equally in a 6-well plate and placed in a CO₂ incubator and they were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ with the medium replaced every 24 h. In the first 48 h, 5-bromodeoxyuridine (0.1 mM) was added to prevent the proliferation of non-myocardial cells. Eventually more than 95% of the cells were myocardial cells as evidenced by identification using an immunohistochemical method with an α-sarcomeric actin antibody.

RNA Intervention
When the cells were grown to almost 80% fusion, siRNA intervention was conducted. The attached cells were diluted with normal medium to 1×10⁶ cells/well. Then 2–8 µl of siRNA transfection reagents A and B were diluted separately to 100 µl with siRNA transfection medium containing no serum or antibiotics, and then were mixed gently, and incubated for 15–45 min at room temperature. The cells were washed once with 2 ml of siRNA transfection medium. For each transfection, 0.8 ml siRNA transfection medium was put into each tube containing the siRNA transfection reagent mixture (solution A + solution B), mixed gently, added to the
culture plate, and incubated for 5–7 h at 37℃ in a CO₂ incubator. Then 1 ml of normal growth medium containing the normal serum was added. The cells were cultured for an additional 18–24 h and the medium was replaced with fresh normal growth medium. After 24–72 h of culture, PHB expression was assayed with western blotting to assess the silencing of PHB siRNA.

**Myocardial Apoptosis**

The cell apoptosis was analyzed by flow cytometry after staining DNA with propidium iodide (PI) and Annexin V-FITC. After treatment with 5 µg/ml of Tan IIA, cells were trypsinized, washed with ice-cold PBS, and fixed with 70% ethanol overnight at −20℃. Then the cells were washed with ice-cold PBS and treated with 200 µl of RNase A (1 mg/ml) at 37℃ for 30–60 min. The cells were then incubated with 800 µl of PI staining buffer (0.1 mg/ml PI and 1% Triton X-100 in PBS) at 4℃ in the dark for 30 min. The cells were analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ).

**Measurement of Mitochondrial Membrane Potential**

Myocardial cells were trypsinized with 0.125% trypsin and diluted to a concentration of 1×10⁵ cells/ml. After washing twice with PBS, they were suspended with Rh123 (100 g/L) which was prepared using 1 g/L Rh123 in DMSO at -20℃ diluted with DMEM without calf serum, and incubated at 37℃, 5% CO₂ for 45 minutes in darkness. Mitochondrial membrane potential was analyzed by flow cytometry (λ_em = 488 nm, λ_ex = 525 nm), and the measured data were processed using CellQuest software. Mitochondrial membrane potential was expressed by the mean fluorescence intensity of the positive cells.

**Measurement of Intracellular Calcium Concentration**

Adherent myocardial cells were trypsinized with 0.125% trypsin and diluted to a concentration of 1×10⁶ cells/ml, and washed twice with PBS. They were loaded with Fluo-3/AM at a final concentration of 10 µmol/L and incubated at 37℃, 5% CO₂ for 30 min in the dark at room temperature. After the cells were attached and washed 2–3 times with PBS, Ca²⁺ responses were detected using a flow cytometer (Becton Dickinson) within 60 minutes (λ_em = 527 nm, λ_ex = 506 nm). The intracellular calcium concentration was calculated by the formula: [Ca²⁺]=Kd [(F-Fmin)/(Fmax-F)]. Kd is the dissociation constant of Fluo-3, i.e., 400 nM; F is the channel fluorescence intensities of each sample; Fmax and Fmin represents the channel fluorescence intensity of each sample added to 0.1% Triton X-100 and 5 mM EGTA, respectively.

**Western Blotting**

Protein concentration was determined by BCA assay and after SDS–PAGE electrophoresis, the protein was transferred to a PVDF membrane. After mounting with TBST (Tris-buffered saline + 0.1% Tween-20) containing non-fat dry milk (5% w/v) for 2 h, the membrane was incubated with 1:800 PHB primary monoclonal antibody overnight at 4℃. After washing with TBST 3 times, the membrane was incubated with HRP-conjugated goat anti-mouse secondary antibody (1:1000) for 1 h at room temperature. After washing 3 times with TBST, the blots were developed using a chemiluminescence detection kit. The image was scanned using a gel image analysis system, and the grey scale values of the experimental points were compared with that of the internal reference -actin, and statistically analyzed with Kodak Software.

**Data Analysis**

The data were expressed as X ± s and analyzed with SPSS13.0 statistical software. One-way ANOVA was used for comparison among many groups of data and the LSD test was used for comparison between two groups. P<0.05 was considered as statistically significant.

**RESULTS**

**Effects of Tanshinone IIA and siRNA on Apoptotic Rate, [Ca²⁺]i and MMP of Myocardial Cells**

In the oxidative stress group, the apoptotic rate and [Ca²⁺]i in the myocardial cells were significantly increased and MMP was significantly decreased as compared with the normal control group (all P<0.05). In the TSN group and the siRNA group, the apoptotic rate, and [Ca²⁺]i significantly increased and MMP in myocardial cells was decreased compared with the model group (all P<0.05). In the siRNA + oxidative stress group, the apoptotic rate and [Ca²⁺]i significantly increased and MMP decreased in myocardial cells as compared with the oxidative stress group as shown in Table 1.
**Table 1.** Effect of tanshinone IIA (TSN) and siRNA on apoptotic rate, \([\text{Ca}^{2+}]_i\) and MMP of myocardial cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptotic rate (%)</th>
<th>([\text{Ca}^{2+}]_i) (nmol/L)</th>
<th>MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>2.53±0.18</td>
<td>94.56±4.51</td>
<td>83.65±1.79</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>44.82±2.43*</td>
<td>277.27±7.81*</td>
<td>47.54±1.34*</td>
</tr>
<tr>
<td>TSN</td>
<td>17.48±1.20▲</td>
<td>133.35±8.64▲</td>
<td>76.53±1.21▲</td>
</tr>
<tr>
<td>siRNA</td>
<td>13.56±1.03▲</td>
<td>133.59±10.90▲</td>
<td>79.32±0.81▲</td>
</tr>
<tr>
<td>siRNA+oxidative stress</td>
<td>58.27±1.23▲</td>
<td>336.65±7.85▲</td>
<td>40.99±2.18▲</td>
</tr>
<tr>
<td>siRNA negative control</td>
<td>2.66±0.13▲</td>
<td>92.22±5.25▲</td>
<td>83.70±0.98▲</td>
</tr>
</tbody>
</table>

**Notes:** Compared with the normal control group, *P*<0.05; Compared with the oxidative stress group, ▲*P*<0.05.

**Effects of Tanshinone IIA and siRNA on PHB Protein Experiment in Myocardial Cells**

The ratio of the grey scale value of PHB to that of β-actin in the normal control group was set as 1, and the ratio was 1.69±0.041 in the oxidative stress group, 1.26±0.058 in the TSN group, 0.57±0.019 in the siRNA group, 0.57±0.019 in the siRNA+oxidative stress group, and 0.99±0.033 in the siRNA negative control group. Statistical analysis indicated that the expression of PHB in myocardial cells in an oxidative stress state increased significantly, which was attenuated by TSN pretreatment. Application of siRNA against prohibitin significantly decreased PHB expression, indicating an obvious gene silencing effect. The expression of PHB was not affected in the siRNA negative control group as shown in Figure 1.

**DISCUSSION**

Coronary artery bypass grafting (CABG), thrombolytic therapy, coronary intervening therapy and extracorporeal circulation all can induce ischemia-reperfusion injury (I/RI). At present, the mechanism of I/RI is not completely clear, but many experimental studies have shown that a large number of oxygen free radicals (active oxygen) produced in reperfusion lead to structure injury, dysfunction and metabolic disturbance of cells, and I/IR not only results in cell necrosis, but also induces cell apoptosis.5-8 Excessive apoptosis can lead to a decrease of cell number, structure destruction and dysfunction of myocardial cells. It is possible that intracellular calcium ion may initiate apoptosis, and inhibition of calcium concentration may prevent the induced apoptosis.9,10 Decrease of MMP occurs in the early period of apoptosis, indicating that the mitochondrial membrane structure has been damaged. In this study, it was determined that exogenous H2O2 at a concentration of 200 mol/L could induce a significant increase in apoptosis rate and \([\text{Ca}^{2+}]_i\), and a significant decrease of MMP. However, after TSN was added, the apoptosis rate and \([\text{Ca}^{2+}]_i\) were significantly decreased, and the MMP was significantly increased, indicating that TSN has a protective effect on myocardial cell apoptosis induced by oxidative stress.

PHB gene was first found as a tumor suppressor gene, and it is also called anti-proliferin because of its significant anti-proliferation and anti-tumor effects.11 Prohibitin protein is localized in the inner mitochondrial membrane functioning as a molecular chaperone, and in the nuclear membrane functioning in transcriptional control.12-14 Natural substrates of PHB include many kinds of proteins, such as cytochrome oxidase and compound I in the mitochondrial oxidation-reduction respiratory chain, among others. Upon disturbance of mitochondrial protein synthesis, PHB increases and plays a role as a molecular chaperone to stabilize various metabolic functions.15 PHB in mitochondria can regulate ion channels and production of calcium-dependent ATP, suggesting that PBH is possibly related to intracellular energy metabolism.16 The relationship between PHB protein and diseases has been studied using...
proteomics.\(^1\) The authors’ previous study confirmed that in ischemia/reperfusion, PHB protein expression was increased in rats, which is in accordance with Kim’s study result.\(^2\) In the present study, it was shown that prohibitin protein helped to resist the myocardial apoptosis induced by H\(_2\)O\(_2\).

RNA interference (RNAi) technique also known as mRNA post-transcription gene silencing (PTGS) works by specific inhibition of specially designated target genes by complementary exogenous small interfering RNA (siRNA).\(^2\) In the present study, after interference of specific siRNA of PHB, PHB expression was not completely inhibited, but the PHB band in the siRNA group had become significantly more thin and light as compared with the normal group, indicating that the PHB expression level was decreased. After the inhibition of prohibitin expression by siRNA, the cell apoptosis rate and [Ca\(^{2+}\)]i significantly increased, and mitochondrial membrane potential significantly decreased as compared with the normal group. The cell apoptosis rate and [Ca\(^{2+}\)]i significantly increased and MMP decreased in the siRNA + oxidative stress group as compared with the oxidative stress group. These results indicated that PHB protein has a protective effect on myocardial cells under oxidative stress similar to another report that PHB has anti-oxidation activities.\(^2\)

TSN, the main component of Danshen (丹参 Radix Salvia miltiorrhiza), is a natural antioxidative reagent and it can effectively inhibit cellular lipid peroxidation.\(^2\) In the present study, the outstanding protective action of TSN on myocardial cells under a state of oxidative stress and the decrease in the level of expression of PHB protein seem to be contradictory. However, the results indicated that the increase of prohibitin protein expression in myocardial ischemia injury may be a compensatory response to oxidative stress.\(^4\) A current study shows that interleukin-6 (IL-6) can stimulate PHB protein expression in enteric epithelium and the regulative effect of IL-6 is controlled by signal transducer and activator of transcription 3 (STAT3).\(^2\) Many studies show that a lot of inflammatory factors participate in the process of ischemia-reperfusion injury, and TSN has anti-inflammatory action and can effectively down-regulate expression of these inflammatory factors, such as IL-6, TNF-\(\alpha\) and so on.\(^2\) In this study, it was determined that the PHB protein level in myocardial cells was down-regulated by TSN pretreatment under conditions of oxidative stress, but it was still higher than that in the normal control group. There may be two reasons for this. First, TSN may have down-regulated PHB by attenuating the oxidative injury of myocardial cells leading to a decrease of prohibitin protein expression. Second, TSN may promote direct expression of PHB protein during oxidative injury. The study of the mechanism of TSN in regulating PHB expression is ongoing.

REFERENCES


(Received April 3, 2010)