Anti-oxidation of Tanshinone II A and Prohibitin on Cardiomyocytes

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Abstract: Objective To investigate the anti-apoptotic mechanism of tanshinone II A and the function of prohibitin (PHB) on myocardial cells apoptosis induced by hydrogen peroxide (H2O2). Methods Myocardial cells were primary cultured neonate rat were cultured in medium with 200 μmol/L H2O2, and the medium was supplemented with tanshinone II A (1 × 10^{-4} mol/L) in advance for 24 h. PHB in myocardial cells was knocked down by RNA interference, and the expression level of PHB was determined by Western blotting analysis. Flow cytometric analysis was used to detect apoptosis rate, intracellular calcium concentration ([Ca^{2+}]_i), and mitochondrial membrane potential (MMP). Results H2O2-mediated cell apoptosis resulted in activation of PHB, increasing of [Ca^{2+}]_i, and decreasing of MMP. Tanshinone II A profoundly inhibited myocardial cell apoptosis induced by H2O2, decreased [Ca^{2+}]_i, and increased MMP. Specific silence of PHB by siRNA down-regulated the expression level of PHB, increased apoptosis rate and [Ca^{2+}]_i, and decreased MMP. Conclusion The results demonstrate that tanshinone II A could attenuate apoptosis induced by H2O2, and the activation of PHB induced by H2O2 is the major regulatory pathway of cyto-protective gene expression against oxidative stress.

Key words: calcium overload; myocardial cell; oxidative stress; prohibitin; tanshinone II A

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Introduction

Ischemia-reperfusion (IR) injury secondary to revascularization with thrombolytic drugs or interventional procedures, such as percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) is an emerging problem (Schäfer et al., 2002). Although timely reperfusion of acute ischemic myocardium is essential for myocardial salvage, reperfusion may also result in a unique form of myocardial damage. A number of mechanisms have been proposed to mediate reperfusion injury, of which perhaps the most important is the formation of oxygen radicals. A large number of studies have shown that free radical scavenger could prevent or scavenge damaging oxidants and free radicals (Sun, 2007).

Tanshinone II A is one of the major lipid-soluble constituents of Salvia miltiorrhiza Bunge known as Danshen (Wang et al., 2008), which has long been used for prevention and treatment of cardiovascular diseases in China (Chen, Lu, and Zheng, 2009). Accumulating studies show that tanshinone II A possesses many biological and pharmacologic properties (Kimm et al., 2004; Du et al., 2005) mainly depending on its anti-oxidative effects (Tang et al., 2007). In our previous study we have shown in a rat model that tanshinone II A could attenuate arrhythmia induced by myocardial IR by inhibition of nitric oxide (NO) and platelet adhesion molecules, such as CD41 and CD62P (Sun, Jia, and Chen, 2000; Jia, Sun, and Chen, 2002). The purpose of this article is to investigate the effect of tanshinone II A on apoptosis induced by hydrogen peroxide (H2O2) in primary cultured rat cardiomyocytes in vitro and underlying molecular mechanisms.

Prohibitin (PHB), a highly conserved protein, is localized in the inner mitochondrial membrane. The function of PHB is associated with stabilization of mitochondrial function, suppression of tumor cell proliferation, regulation of apoptosis and cell proliferation, protection against aging, and so on (Mishra et al., 2005). Recently, we used proteomic analysis to detect differentially

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expressed proteins in IR hearts, we finally identified that PHB was in high expression in the IR hearts compared with the control hearts, the result was consistent with the study by Kim et al. (2006). However, the specific cellular function of PHB in myocardial cells has not been clearly elucidated. The goal of this study was to investigate the anti-apoptotic mechanism of tanshinone II_A, the regulation and the function of PHB during injury induced by H₂O₂. In order to elucidate the function of PHB, RNA interference (RNAi) was used to knock down the expression of PHB.

Materials and methods

Materials

SPF Wistar neonatal rats (1–3 d old) were purchased from Experimental Animal Center of Southern Medical University. Trypsin and DMEM culture media were purchased from Gibico (USA). siPORT NeoFX reverse transfection kit was purchased from Ambion (USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biotech Co., Ltd. Tanshinone II_A was purchased from China Pharmaceutical Biological Products Analysis Institute. PHB antibody, goat antimouse IgG-HRP, and PHB siRNA, and transfection reagent were purchased from Santa Cruz (USA). ECL chemiluminescence kit and protein excretion kit, Fluo-3/AM, Rhodamine 123 (Rh123), and AnnexinV-FITC cell apoptosis detection kit were purchased from Nanjing KeyGen Biotech Co., Ltd.

Primary cardiomyocyte culture

Primary cardiac myocyte culture from neonatal Wistar rats were prepared according to the procedure described by Goldenberg et al. (2003). In brief, the hearts were surgically removed from (1–3) d old Wistar rats, minced into (1–2) mm³ pieces and then washed for three times with D-Hanks. The minced tissue was put into a 50 mL tube containing 0.1% trypsin solution for trypsinization, 37 °C for 6 min. Supernatant was dispersed at first time. Then, the tissue was subjected to seven to ten cycles’ trypsinization, until all the tissues were trypsinized. Supernatants from each cycle were collected and centrifuged twice with DMEM with 5% fetal bovine serum at 1000 r/min. For selective enrichment with cardiac myocytes, dissociated cells were preplated for 1 h, during which period the non-myocytes attached readily to the bottom of the culture dish. In the end, the cell pellet was resuspended in DMEM supplemented with 15% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Bromodeoxyuridine (0.1 mmol/L) was added during the first two days to prevent the proliferation of non-myocytes. The cells were cultured in DMEM containing 15% FBS, 150 mmol/L HEPES, 100 U/mL penicillin, and 100 g/mL streptomycin. Cultured cells were then incubated at 37 °C in humidified atmosphere of 95% air and 5% CO₂, medium was replaced every 24 h. More than 95% of cells were cardiomyocytes, as evidenced by immunostaining with α-sarcomeric actin antibody. For all experiments, myocardial cells were grown to 80%–90% confluence and made quiescent by starvation for 24 h. Tanshinone II_A was added 24 h before treatment with H₂O₂.

Evaluation of cytotoxicity

Cells were cultured in a 96-well microplate. Increasing concentration of H₂O₂ (50, 100, 200, 400, and 800 μmol/L) and tanshinone II_A (1 × 10⁻⁵, 5 × 10⁻⁵, and 1 × 10⁻⁴ mol/L) were added to the culture. After 2 h incubation, cytotoxicity of H₂O₂ was assessed by MTT assay. After 24 h incubation, cytotoxicity of tanshinone II_A was assessed by MTT assay. MTT solution (5 mg/mL) was added to each well in the assay, and plates were incubated at 37 °C for 4 h in the dark. After 4 h, the medium was removed, and 200 μL DMSO was added to each well and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on ELISA plate reader at OD 570 nm.

RNA intervention

Adherent cells were trypsinized and diluted in normal growth medium to 1 × 10⁵ cells per mL. Diluted (2–8) μL of siRNA transfection reagent A and B into 100 μL siRNA transfection medium. Mixed gently by pipetting the solution up and down and incubate the mixture (15–45) min at room temperature. Diluted siRNA appropriately with siRNA dilution buffer. Washed the cells once with 2 mL of siRNA transfection medium. For each transfection, 0.8 mL siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture (solution A + solution B). The mixture was mixed gently and overlay onto the washed cells. The cells were incubated (5–7) h at 37 °C in a CO₂ incubator. Normal growth medium (1 mL) containing two times the normal serum and antibiotics
concentration was added. The cells were incubated for an additional (18–24) h. The medium was aspirated and replaced with fresh 1 × normal growth medium. The effects of RNAi were assayed by Western blotting after the addition of fresh medium in the step above.

Apoptosis analysis

The cell apoptosis was analyzed by flow cytometry after staining DNA with propidium iodide (PI) and Annexin V-FITC. At the indicated time points after 5 μg/mL of Tanshinone II_A treatment, cells were trypsinized, rinsed with ice-cold PBS, and fixed in 70% ethanol for overnight at −20 °C. After the fixation, cells were washed with ice-cold PBS and treated with 200 μL of RNase A (1 mg/mL) at 37 °C for (30–60) min. 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 °C in the dark for 30 min. Afterwards, cells were analyzed by FACS on a Calibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ).

Measurement of MMP

Myocardial cells were trypsinized and harvested, and diluted to a concentration of 1 × 10^5 cells per mL. After washing the cells twice in PBS, they were loaded with Rh123 (100 μg/L), and incubated at 37 °C, 5% CO₂ for 45 min in darkness. MMP was analyzed by flow cytometry (λ_em = 488 nm, λ_ex = 425 nm), and fluorescence intensities were measured using Cell Quest software. MMP was expressed by mean fluorescence intensities of the positive cells.

Intracellular calcium measurement

Adherent cells were trypsinized and harvested, and diluted to a concentration of 1 × 10^5 cells per mL. After washing the cells twice in PBS, they were loaded with Fluo-3/AM at a terminal concentration of 10 μmol/L, and incubated at 37 °C, 5% CO₂ for 30 min in darkness. Then cells were washed twice in PBS, Ca^{2+} responses were observed and evaluated by means of flow cytometry (FACS sort, Becton Dickinson) within 60 min (λ_em = 527 nm, λ_ex = 506 nm). Mean channel fluorescence intensities were calculated using Cell Quest software. Intracellular calcium concentration ([Ca^{2+}])_i was calculated by the formula:

\[ [Ca^{2+}]_i = k_d \left( F - F_{min} \right) / \left( F_{max} - F \right) \]

where \( k_d \) represents dissociation constant of Fluo-3, the value is 400 mmol·L⁻¹·s⁻¹, \( F \) represents channel fluorescence intensities of each sample, \( F_{max} \) and \( F_{min} \) represents channel fluorescence intensities of each sample that was loaded with 0.1% Triton X-100 or 5 mmol·L⁻¹ EGTA, respectively.

Western blotting

Protein concentration was determined by BCA protein assay kit. Protein (50 μg) was subjected to 10% SDS–PAGE, and then transferred to nitrocellulose membrane. After 1 h blocking with non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated with 1 : 800 primary antibody for PHB overnight at 4 °C. After washing, the membrane was incubated with HRP-conjugated goat anti-mouse secondary antibody (1 : 1000) for 1 h at room temperature. After washing, the blots were developed using an enhanced chemiluminescence detection kit. The membrane was then reprobed with β-actin antibody. The blotted was quantified by densitometry and normalized by using β-actin signal in order to correct any error during sample preparation and protein loading. The intensity of bands obtained from Western blotting images were captured and estimated with Kodak software.

Statistical analysis

The data presented as the means ± SEM. Statistical analysis was made by one-way ANOVA followed by LSD test. A value of \( P < 0.05 \) was considered statistically significant.

Results

Effects of tanshinone II_A on H₂O₂-induced injury in myocardial cells

Myocardial cells were exposed to various concentration of H₂O₂ (50–800) μmol/L for 2 h, and the cell cytotoxicity was determined by MTT assay. As shown in Fig. 1A, H₂O₂ significantly inhibited the viability of myocardial cells in a dose-dependent manner. Additionally, cell viability was significantly improved in the presence of tanshinone II_A in a dose-dependent manner (Fig. 1B).

Effect of tanshinone II_A and siRNA on cell apoptosis related changes

To clarify whether the effect of tanshinone II_A (1 × 10⁻⁴ mol/L) was related to the alteration of myocardial cell apoptosis status, we examined cell apoptosis rate, [Ca^{2+}]_i, and MMP. As shown in Table 1, apoptosis rate and [Ca^{2+}]_i were significantly increased, and MMP levels were significantly down-regulated by treatment with H₂O₂ (200 μmol/L) for 2 h. Pretreatment with tanshinone II_A (1 × 10⁻⁴ mol/L) for 24 h provided significant protection.
Figure 1 Myocardial cells treated with H_2O_2 and tanshinone II_A at different concentration
A: Myocardial cells treated with 50, 100, 200, 400, and 800 μmol·L^{-1} H_2O_2 for 2 h and pretreated
B: Myocardial cells pretreated by 1 × 10^{-5}, 5 × 10^{-5}, and 1 × 10^{-4} mol·L^{-1} tanshinone II_A for 24 h before addition of H_2O_2
*P < 0.05 vs control group
**P < 0.05 vs oxidative stress group

Both cell cytotoxicities by H_2O_2 and the protective effect by tanshinone II_A were assessed by the MTT assay, and the absorbance was read at 570 nm against down-regulation of apoptosis rate, [Ca^{2+}]_i caused by H_2O_2, while the MMP levels were significantly up-regulated. siRNA against PHB treatment significantly increased apoptosis rate and [Ca^{2+}]_i, and decreased MMP in myocardial cells, the values were more noticeable in siRNA+ oxidative stress group.

Effects of tanshinone II_A and siRNA on PHB protein expression in myocardial cells
In this study, we set the ratio of the gray value between PHB protein and β-actin as 1, then the ratio was 1.691 ± 0.041 in oxidative stress other groups, 1.261 ± 0.058 in tanshinone II_A group, 0.553 ± 0.020 in siRNA group, 0.576 ± 0.019 in siRNA+ oxidative stress group, and 0.992 ± 0.033 in negative control siRNA. The expression of PHB increased remarkably under oxidative stress conditions (Fig. 2). However, H_2O_2-induced increase in the quantity of PHB was attenuated by tanshinone II_A pretreatment compared with oxidative stress group. siRNA against PHB treatment specifically decreased PHB protein, indicating that siRNA was effective in down-regulating the protein expression. The quantity of PHB was not affected by negative control siRNA.

Table 1 Effect of tanshinone II_A and siRNA against PHB on H_2O_2-induced changes in myocardial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Apoptosis rate/ %</th>
<th>[Ca^{2+}]_i / (mmol·L^{-1})</th>
<th>MMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>2.530±0.183</td>
<td>94.560±4.511</td>
<td>83.650±1.793</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>44.820±2.430*</td>
<td>2772.73±7.818*</td>
<td>47.543±1.341*</td>
</tr>
<tr>
<td>tanshinone II_A</td>
<td>17.477±1.96▲</td>
<td>133.350±8.635▲</td>
<td>76.530±1.206▲</td>
</tr>
<tr>
<td>siRNA</td>
<td>13.560±1.02▲</td>
<td>133.593±10.903▲</td>
<td>79.320±0.805▲</td>
</tr>
<tr>
<td>siRNA + Oxidative stress</td>
<td>58.273±1.225*</td>
<td>336.648±7.852*</td>
<td>40.986±2.182*</td>
</tr>
<tr>
<td>negative control siRNA</td>
<td>2.657±0.125*</td>
<td>92.217±5.249</td>
<td>83.703±0.981</td>
</tr>
</tbody>
</table>

*P < 0.05 vs normal control group; ▲P < 0.05 vs oxidative stress group

Figure 2 Effect of tanshinone II_A on H_2O_2-induced changes in PHB protein and siRNA against prohibitin
1: The quantity of PHB protein in normal control group
2: The quantity of PHB protein in oxidative stress group
3: The quantity of PHB protein in tanshinone II_A group
4: The quantity of PHB protein in siRNA group
5: The quantity of PHB protein in siRNA + oxidative stress group
6: The quantity of PHB protein in siRNA negative control group

Discussion
IR injury is a well-known phenomenon following CAGB, thrombolytic therapy and cardiopulmonary bypass therapy (CPBT). A number of mechanisms have been proposed to mediate reperfusion injury, of which the most important is the formation of reactive oxygen species (Wang et al., 2008; Giacomo and Antonio, 2007; Fischer et al., 2006). Myocardial IR injury not only results in cell necrosis, but also induces cell apoptosis (Bognar et al., 2006) which can be triggered through different mechanisms in response to both intracellular and extracellular signals. The excessive apoptosis leads to the decreasing of cell number, damages of cell structure and cell function. There is strong evidence that...
elevated \([\text{Ca}^{2+}]\), is the initial factor of apoptosis (Sodha et al., 2008) and inhibition of calcium concentration may prevent the process of apoptosis (McAinsh and Pittman, 2009). MMP decreasing occurs in the early period of apoptosis, which means that the mitochondrial membrane has been damaged. In this study, we found that exogenous \(\text{H}_2\text{O}_2\) at a concentration of 200 \(\mu\text{mol/L}\) was associated with a remarkable increase of apoptosis rate and \([\text{Ca}^{2+}]\), and a significant decrease of MMP. In this study, we found that tanshinone \(\text{II}_\alpha\) inhibited the alteration of \([\text{Ca}^{2+}]\), and MMP induced by \(\text{H}_2\text{O}_2\), so that cell apoptosis rate was remarkably reduced. Therefore, tanshinone \(\text{II}_\alpha\) was proved to have protective effect on myocardial cells against apoptosis induced by oxidative stress.

PHB was first found as a tumor suppressor gene, for its significant anti-proliferative activity (Galang, Sasaki, and Maulik, 2000), so it is also called anti-proliferative protein. PHB protein is evolutionally conserved, it is localized in the inner mitochondrial membrane (Dell'Orco et al., 1996). The nuclear localization of PHB has also been reported in a variety of cell lines (Czarnecka et al., 2006; Winter, Kämäräinen, and Hofmann, 2007). A variety of functions of PHB have been suggested, including a role in cell cycle regulation, differentiation, and controlling apoptosis. The natural substrates of PHB include cytochrome oxidase, mitochondrial complex I, and so on. PHB proteins also play crucial roles in the mitochondria. Recently, human PHB1 was reported to associate with mitochondrial complex I, suggesting a regulation of mitochondrial respiratory activity. PHB has been shown to function as chaperones that stabilize newly synthesized mitochondrial protein (Kasashima et al., 2006). PHB has also participated in the production of calcium-dependent ATP, suggesting that PHB plays a role in regulation of energy metabolism (Artal-Sanz et al., 2003). Currently, the finding of the relationship between PHB protein and diseases mostly studied by proteomics (Ferrer et al., 2007; Saridaki and Panayotou, 2005; Hsieh et al., 2006; Li et al., 2004; Mi et al., 2006). Our previous study confirmed that IR injury led to a increase of protein in rats, the result is in accordance with Kim’s study (Kim et al., 2006). The present study is that PHB protein shows an anti-apoptosis effect against \(\text{H}_2\text{O}_2\)-induced injury.

RNAi is a process of sequence specific degradation of RNA in the cytoplasm of eukaryotic cells that is induced by double stranded RNA, and causes target RNA post-transcription gene silencing (Gonzalez-Gonzalez, Lopez-Casas, and Del, 2008). Currently, the RNAi technology is widely utilized in the functional genomic studies and intense research is being carried out around the world to exploit siRNA for therapeutic purposes. In the present study, we have evaluated the potential of specific PHB siRNA to inhibit PHB protein expression in myocardial cells. As a result, a remarkable inhibition of PHB was observed after siRNA against PHB treatment, although the inhibition was not totally, and was not as satisfactory as we thought. The reason may be that siRNA transfection efficiency varied from one cell type to another, it is not effective in primary cultured myocardial cells. Anyway, the inhibition of PHB by siRNA caused a significant increase in cell apoptosis rate and \([\text{Ca}^{2+}]\), and a decrease in MMP, the changes were more remarkable in siRNA + oxidative stress group. This result indicated that PHB proteins show protective effect against oxidative stress. A recent report demonstrated that PHB played an important role in combating oxidative stress in human and animal models of inflammatory bowel disease (Theiss et al., 2007). Our study shows the similar function of anti-oxidative activity of PHB.

Free radicals have been suggested as potentially important causative agents of aging and several human diseases. Therefore, one of the strategies is to look for natural products from plants for combating free radical-induced pathological status. Tanshinone \(\text{II}_\alpha\) is one of the major lipophilic components of the herbal medicine *S. miltiorrhzia*. A variety of studies show that tanshinone \(\text{II}_\alpha\) is a natural antioxidative drug against lipid peroxidation (Zhang et al., 2003; Zhang et al., 2004). In the present study, the outstanding anti-oxidative activity of tanshinone \(\text{II}_\alpha\) was observed in myocardial cells. Besides, a decrease in the amount of PHB protein was also observed after pretreatment of tanshinone \(\text{II}_\alpha\). The result seems paradox, as PHB protein proved to be beneficial for myocardial cells against oxidative stress. Actually, that is not the case. The increase of PHB protein is a compensatory response to the oxidative stress (Kim et al., 2006). A current study showed that the acute phase cytokine interleukin-6 (IL-6) increased PHB protein and mRNA abundance and induced PHB promoter.
activation, and IL-6 responsiveness are affected by signal transducer and activator of transcription 3 (STAT3). Many studies support that a lot of inflammatory factors participate in the process of IR (Podgoreanu et al., 2005), and tanshinone IIA could effectively down-regulate the amount of these inflammatory factors (Jang et al., 2003), such as IL-6, TNF-α, and so on. In this study, we found that the level of PHB protein level in myocardial cells was down-regulated by tanshinone IIA pretreatment under oxidative stress condition, while the amount of the protein was still larger than that in the normal control group. There may be two reasons for this case. First, tanshinone IIA probably down-regulated PHB by attenuating oxidation reaction that subsequently led to a decrease in the amount of PHB protein. Second, tanshinone IIA may down-regulate PHB directly. In this case, we hypothesize that tanshinone IIA down-regulates the expression of PHB by inhibiting the activation of inflammatory factors in myocardial cells under oxidative stress or acting directly on PHB. As for the real mechanism, we need further studies to confirm this suppose.

Reference


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