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Original Article

The molecular biological mechanism of tanshinone VI on the treatment of the myocardium diseases

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Abstract: The tanshinone VI (the structure is shown in Figure 1) has recently attract various attentions because it is very effective for the clinical angina pectoris. In this paper, the molecular biological mechanism of its influence on the regulation of PLM to the ion channels as well as the hormone level was investigated by Western blotting method. The cardiac muscle cells were cultured with tanshinone VI in different environments and then transfected by PLM-pAdtract. The overexpression of phosphorylation sites were analyzed by Western blotting. The results show that the tanshinone VI could efficiently affect the phosphorylation of Ser68 and Ser63. Moreover, in harsh conditions, the tanshinone VI could simultaneously restrain the expression of ERK 1/2 and increase the expression of phosphorylated Survivin. As a result, in the oxygen deprivation and glucose deprivation conditions, tanshinone VI is not only benefit to the myocardial contractive power via affecting the ion channels, but also to the enhancement of the cell survive via affecting the hormone protein expression.

Keywords: Tanshinone VI, western immunoblots, phospholemman (PLM), ERK 1/2, survivin

Introduction

In 2000, a group of ion channel regulation proteins were firstly discovered by Sweadner and Rael, naming as FXYD, due to the PFXYD sequence locating at the end of the N in these proteins [1]. A total of 12 species of these proteins have been found, including FXYD1 (Phospholemman PLM), FXYD2 (γ subunit of Na^+, K^+-ATPase), FXYD3 (mammary tumor marker 8, mat-8), fxyd4 (corticosteroid hormone-induced factor, CHIF), FXYD5 be related to ion channel RIC), FXYD6 phosphohippolin, FXYD7 and FXDY10 (the shark homolog of PLM). The FXYD protein was mainly distributed in the tissues and organs with function of solute and fluid regulation, such as kidney, intestine, breast, pancreas, liver, etc. They also exist in the tissues and organs with characteristics of the electrical activity such as heart, skeletal muscle and brain. The FXYD protein contains at least one of the serine or threonine, indicating potential phosphorylation sites on the FXYD protein.

The Phospholemman (PLM), i.e. FXYD1, is a kind of cross-membrane phosphorylation protein with 72 amino acids [2]. A total of 17 amino acids on the N-terminal are located at outside surface of the membrane, while 35 amino acids on the C-terminal are located internal surface of the membrane. The cross-membrane segment consists of 20 amino acids, including the PFXYD sequence at 8-12 positions. PLM is widely distributed in the myocardial cell membrane, which is the main phosphorylation substrate for cardiac muscle cells. The C terminus on the inner side of the membrane contains 4 phosphorylation sites, including Ser62, Ser63, Ser68, and Thr69 [3]. It has been found that the Ser68 could be phosphorylated by Protein Kinase A (PKA) in vivo, while the Ser68 and Ser63 could be phosphorylated by Proteins Kinase C (PKC). The in vitro study also confirmed that the phosphorylation of Ser63 by PKA and the phosphorylation of Thr69 by PKC [4]. In recent years, the role of PLM becomes a hot topic in cardiac pathophysiology research. The early study found that PLM could be con-

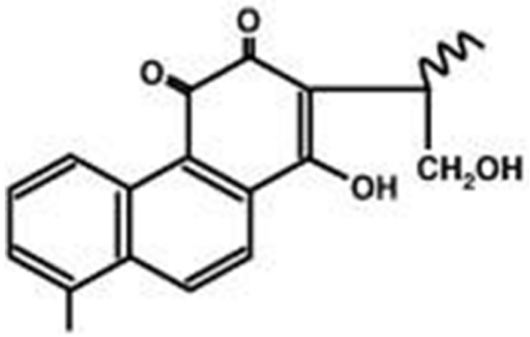

Figure 1. Chemical structure of tanshinone VI.

nected with the Na^+/K^+-ATP enzyme (Na^+/K^+-ATPase, NKA) on myocardial cell membrane and regulate its dynamic characteristics [5-7]. The activity of NKA could be suppressed by PLM, leading to the increase of intracellular Na^+ concentration. The forward transport of Na^+/Ca^{2+} exchanger (NCX), i.e. Ca^{2+} efflux, was decreased while the anti-transport (Ca^{2+} internal flow) enhanced, resulting in increasing of the intracellular Ca^{2+} concentration, as well as the enhancement of the myocardial cell contraction force, which consistent with the positive effect mechanism of the digitalis on the contraction force of myocardial cell. Further studies found that the Ser68 phosphorylation could separate the NKA with PLM, thus relieving the inhibition effect of PLM on NKA [6, 8]. In 2005, Tucker et al successfully produced PLM gene knockout mouse [9-11]. The results of series works showed that PLM knockout mice have stronger myocardial contractility than those of wild-type mice, which is conflict with the theory extrapolation. Hence, it is suggested that PLM may regulate the other channels of ions.

The regulation of PLM to the ion channels could be also influenced by the physiological environment and various drugs. It is reported that phosphorylation of Ser68 could be initiated by adrenaline β receptor agonists, weakening the inhibition of NKA by PLM. Thus, the NKA activity increased, leading to a decrease of intracellular Na^+ concentration. As a result, NCX cotransport (Ca^{2+} efflux) enhanced and intracellular Ca^{2+} concentration reduced, weakening the cardiac myocyte contractility [12].

Another drug for treatment of coronary heart disease, tanshinone VI (the structure is shown in **Figure 1**) has recently attract various attentions because it is very effective for the clinical angina pectoris. However, there is rare contribution devoted to the molecular biological mechanism of its influence on the regulation of PLM to the ion channels. In this paper, cardiac muscle cells were cultured with tanshinone VI in different environments and then transfected by PLM-pAdtract. The overexpression of phosphorylation sites were analyzed by Western blotting.

Experimental

Animals and cell culture

The mice (3 weeks old, weighing 20-25 g) were purchased from Beijing Biocytogen (Beijing, China). A total of 1200 U heparin sodium was intraperitoneally injected to the mice for anticoagulation. 15 minutes later, pentobarbital sodium (30 mg/kg) were intraperitoneally injected. After the anaesthesia, a thoracotomy on mice was immediately performed to take out the heart, which was put into calcium-free Tyrode's solution. The heart was connected onto a Langerdoff irrigation flow device via aortic cannula for perfusion (37°C, 95% O_2). The calcium-free Tyrode's solution was firstly applied for the perfusion, and 10 min later switched to the calcium-free Tyrode's solution with 0.5 mg/ml collagenase II and 1 mg/ml bovine serum albumin. The perfusion were proceed until the expansion, soften, and whitening of the heart. The myocardial tissue was taken out from the free wall of the left ventricular, and then fully shredded for stirring. The obtained individual myocardial cells were inoculated onto a slide covered with laminin. After 2 hours, the cell culture fluid containing tanshinone VI replaced in order to remove the myocardial cells which could not affixed on the slide. For the control group, the myocardial cells were cultured in the cell culture fluid without tanshinone VI.

The similar procedures were also performed in oxygen deprivation (OD) and glucose deprivation (GD) environments.

Preparation of PLM adenovirus vector

The full length PLM-cDNA was subcloned into pAdTrack-CMV vector plasmid with green fluorescent protein (GFP). The PLM-cDNA and padtrack CMV plasmid vector has the same

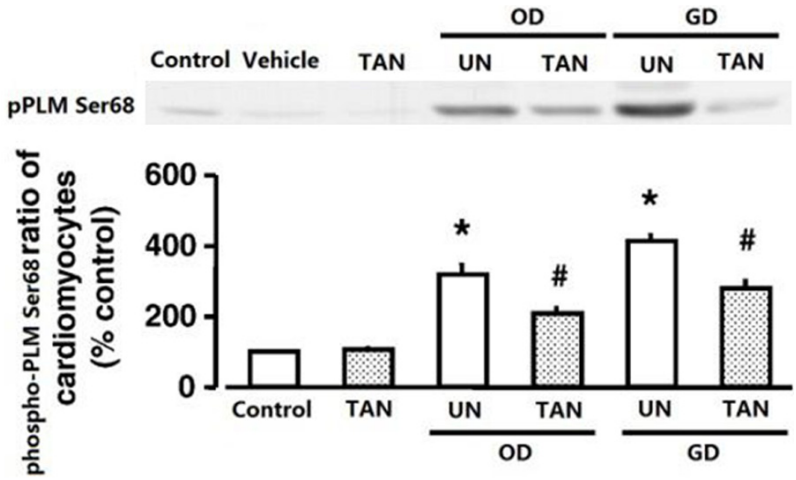

Figure 2. Representative Western blots of phosphorylated PLM (Ser68) and effects of tanshinone VI (Tan) on PLM phosphorylation in cultured cardiomyocytes. Each value represents the mean ± S.E.M. of 5 experiments. Significantly different from control group (Control, $P < 0.05$). #Significantly different from the corresponding tanshinone VI-untreated groups ($P < 0.05$). OD: Oxygen deprivation; GD: glucose deprivation.

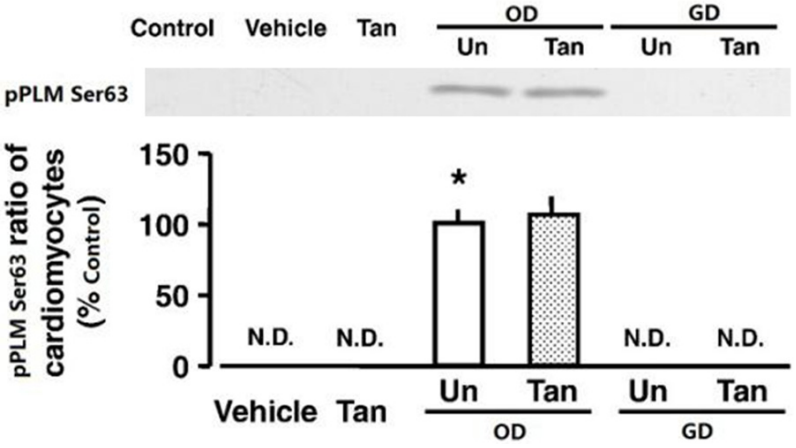

Figure 3. Representative Western blots of phosphorylated PLM (Ser63) and effects of tanshinone VI (Tan) on the PLM phosphorylation in cultured cardiomyocytes. Each value represents the mean ± S.E.M. of 5 experiments. Significantly different from un-treated group (Untreated, $P < 0.05$). #Significantly different from the corresponding tanshinone VI-untreated groups ($P < 0.05$). OD: Oxygen deprivation; GD: glucose deprivation.

restriction enzyme sites: Hind III and EcoR I. The enzyme-digested products were combined by PCR and then translated to the stb13 competent colibacillus cells. The transformed bacteria were cloned in a LA dish containing ampicillin and rice rhzomorph. The single colony was picked out for Amplification, Restriction enzyme analysis, PCR, and DNA sequencing. Once constructed, the recombinant vector (PLM-pAdtract) was transfected into DH5 cells to amplify and purify the target plasmid.

Western blotting

For the Western blotting, the procedure was briefly described as follows: The myocardial cells 72 hours after transfection were harvested and dissolved in the Cell lysis solution for the extraction of total protein. After protein separation by SDS-PAGE electrophoresis, the gel protein was transferred to PVDF membrane. PVDF membrane was sealed in sealing fluid for two hours, followed in sequence by incubation overnight (4°C) with first antibody and with secondary antibody at room temperature for 1 h. After each incubation procedure the PVDF membrane were washed three times in TBST for 10 min. Finally, an ECL kit was applied for protein visualization. The protein band density was quantitative analyzed by image analysis software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data were expressed as the mean 5 standard deviation. The statistical significance was determined by using the Student t test for differences between two groups and one-way ANOVA for differences among multiple groups. Error bars were expressed as standard deviation. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

Results and discussions

To investigate the effect of tanshinone VI stimuli on Ser68, we investigated Ser68 levels in

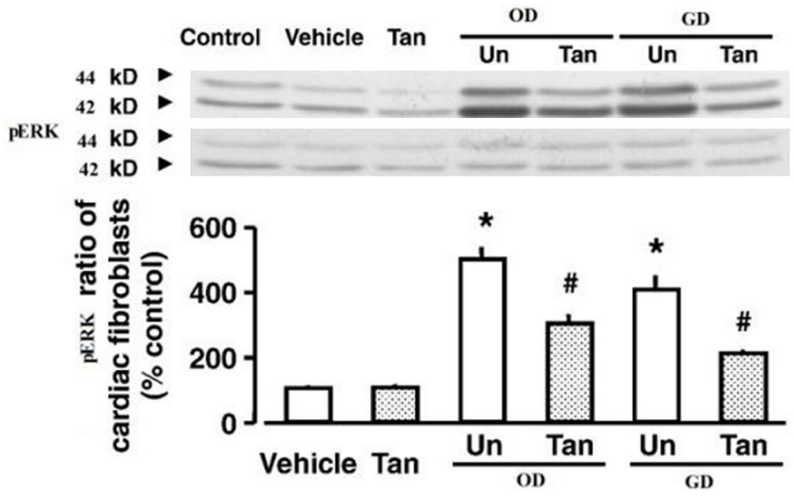

Figure 4. Representative Western blots (upper panels) of phosphorylated ERK 1/2 (pERK) and ERK 1/2 (ERK) and effects of tanshinone VI (Tan) on the ERK phosphorylation in cultured cardiomyocytes (lower panel). Each value represents the mean ± S.E.M. of 5 experiments. Significantly different from control-treated group (Control, $P < 0.05$). #Significantly different from the corresponding tanshinone VI-untreated groups ($P < 0.05$). OD: Oxygen deprivation; GD: glucose deprivation.

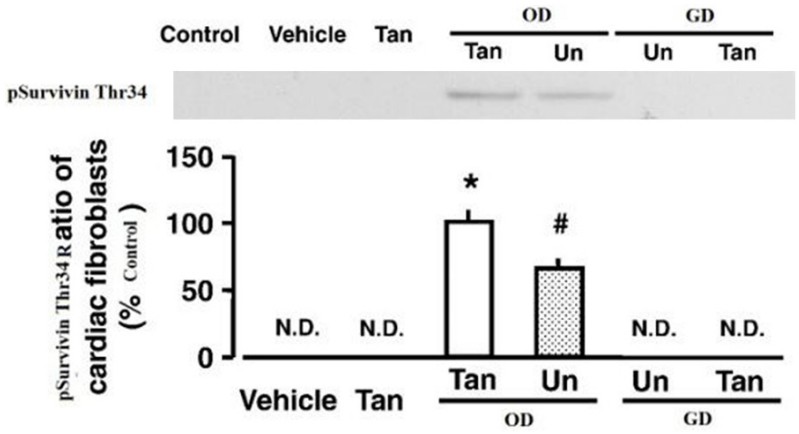

Figure 5. Representative Western blots of phosphorylated Survivin (Thr34) and effects of tanshinone VI (Tan) on the Survivin phosphorylation in cultured cardiomyocytes. Each value represents the mean ± S.E.M. of 5 experiments. Significantly different from un-treated group (UNtreated, $P < 0.05$). #Significantly different from the corresponding tanshinone VI-untreated groups ($P < 0.05$). OD: Oxygen deprivation; GD: glucose deprivation.

cultured myocardial cells in different environments. Representative Western immunoblots of phosphorylated PLM (Ser68) and effects of tanshinone VI (Tan) on PLM phosphorylation in cultured cardiomyocytes were shown in **Figure 2**. Each value represents the mean ± S.E.M. of 5 experiments. For the cells in common environment, the Ser68 expression with tanshinone VI was slightly higher than that in control group. There is no obvious effect of the tanshinone VI on the Ser68 expression. For the control group, the Ser68 expressions were much enhanced in the condition of oxygen deprivation and glucose deprivation. Nevertheless, the Ser68 expression was significantly restrained by tanshinone VI.

Figure 3 shows the representative Western immunoblots of phosphorylated PLM (Ser63) and effects of tanshinone VI (Tan) on the PLM phosphorylation in cultured cardiomyocytes. Each value represents the mean ± S.E.M. of 5 experiments. In the common environment or in case of glucose deprivation, the Ser63 was nearly not phosphorylated whether with tanshinone VI or not. The expression of phosphorylated Ser63 was stronger initiated in the condition of oxygen deprivation, which momentary guarantee the function of the heart in the case of inadequate oxygen supply. It can be seen in **Figure 3** that the tanshinone VI could further increase the expression of phosphorylated Ser63, and thus have a positive effect for hypoxia induced cardiac disease.

Figure 4 shows the representative Western immunoblots (upper panels) of phosphorylated ERK 1/2 (pERK) and ERK 1/2 (ERK) and effects of tanshinone VI (Tan) on the ERK phosphorylation in cultured cardiomyocytes (lower panel). Each value represents the mean ± S.E.M. of 5 experiments. As the ERK 1/2 is quite important in the process of the apoptosis, the influence of tanshinone VI on the expression of phosphorylated ERK 1/2 (pERK) and ERK 1/2 (ERK) may

not only important for the myocardial contraction, but also for the myocardial survive.

It can be seen in **Figure 4** that the expression of and ERK 1/2 is in accordance with that of Ser68. In the common environment, the expression of ERK 1/2 is in ordinal value and the tanshinone VI nearly have no effect. In the oxygen deprivation and glucose deprivation environments, the expression of and ERK 1/2 is much enhanced, leading to a sharp apoptosis, which may threat to life. It is obviously that the tanshinone VI could effectively restrain the expression of ERK 1/2 and thus restrain the apoptosis. Hence, tanshinone VI is and specific medicine for the prevention of the myocardial necrosis in the harsh conditions.

Another hormone protein, Survivin, has opposite effect to the ERK 1/2. It is very important for anti-apoptosis. Representative Western immunoblots of phosphorylated Survivin (Thr34) and effects of tanshinone VI (Tan) on the Survivin phosphorylation in cultured cardiomyocytes are shown in **Figure 5**. Similar with Ser63, the phosphorylated Survivin was nearly not expressed in the common environment or in case of glucose deprivation. In the condition of oxygen deprivation, the expression of phosphorylated Survivin was stronger initiated and the tanshinone VI could further increase the expression of phosphorylated Survivin, which also have a positive effect for hypoxia induced cardiac disease.

Conclusions

In summary, cardiac muscle cells were cultured with tanshinone VI in different environments and then transfected by PLM-pAdtract. The overexpression of phosphorylation sites were analyzed by Western blotting. The tanshinone VI could efficiently affect the phosphorylation of Ser68 and Ser63. Moreover, in harsh conditions, the tanshinone VI could simultaneously restrain the expression of ERK 1/2 and increase the expression of phosphorylated Survivin. As a result, in the oxygen deprivation and glucose deprivation conditions, tanshinone VI is not only benefit to the myocardial contractive power via affecting the ion channels, but also to the enhancement of the cell survive via affecting the hormone protein expression.

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Disclosure of conflict of interest

None.

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