Tanshinone IIA Inhibits miR-1 Expression through p38 MAPK Signal Pathway in Post-infarction Rat Cardiomyocytes

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Key Words

Tanshinone IIA • Myocardial infarction • miR-1 • p38 MAPK • SRF • MEF2 • Cx43

Abstract

Tanshinone IIA is a fat-soluble pharmacologically active ingredient of *Danshen*, a well-known traditional Chinese medicine used for cardiovascular diseases such as coronary heart disease. Tanshinone IIA has been confirmed to suppress miR-1 and reduce the arrhythmogenesis after myocardial infarction (MI). However, the modulation mechanism is not clear. Tanshinone IIA was administrated daily for 7 days before ligation of the left anterior descending artery (LAD) and lasted for 3 months after LAD. Neonatal cardiomyocytes were exposed to 2% O₂+95% N₂ condition for 24 h to simulate ischemia *in vivo*. Protein expression was examined with Western blot and miR-1 level was quantified by Real-time PCR. Our results showed that tanshinone IIA relieved ischemia-induced injury by improving the cardiac function. This beneficial effect may due to the depression of the elevated miR-1 level in ischemic and hypoxic cardiomyocytes, which subsequently restored its target Cx43 protein. Furthermore, tanshinone IIA could

inhibit activated p38 MAPK and heart special transcription factors SRF and MEF2, in ischemic and hypoxic cardiomyocytes. Pretreatment with p38 MAPK inhibitor, SB203580 (10 uM), significantly relieved hypoxia-induced miR-1 increment and restored its downstream target Cx43 protein expression. These data suggest that tanshinone IIA play a role in protection cardiomyocytes from ischemic and hypoxic injury. The effect is based on inhibiting miR-1 expression through p38 MAPK signal pathway. This might provide us a new target to explore the novel strategy for ischemic cardioprotection.

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Introduction

Myocardial infarction is often accompanied by ventricular remodeling, cardiac hypertrophy and cardiac dysfunction, which is one of the risk factors of sudden cardiac death. Our previous study has disclosed that miR-1 expression was significantly up-regulated in post-MI hearts of both patients and rats. And this upregulation produced arrhythmogenic effects to aggravate ischemic injury through targeting the ion channels critical to cardiac

conduction [1]. In another study by Terentyev et al. [2], it has demonstrated that overexpression of miR-1 in cardiomyocytes caused spontaneous arrhythmogenic oscillations of intracellular Ca^{2+} by targeting a Ca^{2+} handling protein. Hence, miR-1 has been considered a new target for antiarrhythmic therapy.

Recently, many researches have suggested that p38 mitogen-activated protein kinase (p38 MAPK) play important roles in myocyte contractile dysfunction, apoptosis, and cell death during ischemia in adult and primary cultured neonatal cardiomyocytes where cardiac-specific activation of the α isoform of p38 MAPK [3-4]. Further study revealed that the application of special p38 MAPK pharmacological inhibitors, such as SB203580, prevented cardiomyocytes from damages and death [5].

Tanshinone IIA (Tan) is a fat-soluble pharmacologically active ingredient of *Danshen* which is isolated from the rhizome of the Chinese herb *Salvia miltiorrhiza* (Fig. 1), a well-known traditional Chinese medicine used for the treatment of cardiovascular diseases such as coronary heart diseases [6-7]. Accumulated evidences suggested that tanshinone IIA reduce ischemic area and improve cardiac function [8-9], and our previous work has identified that tanshinone IIA protected cardiomyocytes by inhibiting elevated miR-1 expression during myocardial infarction [10]. However, how tanshinone IIA downregulating miR-1 expression remains unclear. The present study was designed to shed light on this issue in a rat model of chronic MI (3 months) and in hypoxic neonatal rat cardiomyocytes.

Materials and Methods

Materials

Tanshinone IIA (purity 99%) was purchased from Xian Guanyu Bio-tech Co. Ltd, China. SB203580 was purchased from Sigma, USA. They were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO did not exceed 0.1%. P-p38 MAPK and t-p38 MAPK antibodies were from Cell Signaling Technology (Beverly, MA); Antibodies for SRF, MEF2 and Cx43 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); GAPDH antibody was from Research Diagnostics (Concord, MA).

Rat Models of Myocardial Infarction

Male Wistar rats weighing 220–250 g were randomly divided into three groups: control group, myocardial infarction (MI) group, and tanshinone IIA (Tan) group. Myocardial infarction model was established as described before [1, 11]. Briefly, animals were pretreated with tanshinone IIA (20 mg/kg/d) for 7 days. Rats were anesthetized with 10% chloral hydrate (3 ml/kg) delivered intraperitoneally. All surgical procedures

were performed under sterile conditions. After surgery, survival rats were housed at ambient temperature of 22°C. Tanshinone IIA (20 mg/kg/d) was intragastric administrated daily. 3 months later, the animals' heart was removed and border zone was dissected for use. Control animals underwent open chest procedures without coronary artery occlusion. The use of animals was in accordance with the regulations of the ethics committee of Harbin Medical University.

Measurements of Hemodynamic Parameters

Hemodynamic measurements were obtained before the heart was removed from sham-operated rats ($n = 8$), 3-month MI rats ($n = 6$), and 3-month MI rats pretreated with tanshinone IIA ($n = 6$). A 2-F micromanometer-tipped catheter was inserted through the right carotid artery into the aorta and subsequently advanced to the LV. LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), peak positive and peak negative first derivative of LV pressure ($+dp/dt$ and $-dp/dt$) were calculated as previously reported by averaging 10 consecutive cycles.

Real-Time PCR

The total RNA samples were extracted using Trizol from heart tissues or neonatal myocytes. MiR-1 level was quantified by the mirVana qRT-PCR miRNA Detection Kit (Ambion) in conjunction with Real-time PCR with SYBR Green I, as delineated in detail [1, 12]. The RT primers:

miR-1: 5'GTC GTA TCC AGT GCG TGT CGT GGA GTC GGC AAT TGC ACT GGA TAC GAC TAC ATA C3'; U6: 5' CGC TTC ACG AAT TTG CGT GTC AT 3'. After circle reaction, the threshold cycle (Ct) was determined and relative miR-1 levels were calculated based on the Ct values and normalized by U6 level in each sample.

Western Blot Analysis

For Western blot analysis, 50 μg proteins were fractionated on a 10% SDS- polyacrylamide gel. After electrophoretically transferred to a Pure Nitrocellulose Blotting membrane (Pall Life Science), the blots were probed with primary antibodies, with GAPDH as an internal control. The immunoreactivity was detected using odyssey software (Infrared Imaging System LI-COR Biosciences).

Isolation and Culture of Ventricular Myocytes

Primary cultures of neonatal rat cardiomyocytes were prepared from 1 to 3-day-old Wistar rats by trypsin as described previously [12]. The ventricular myocardium were minced in DMEM, after each of six successive 6 min incubations, the cells were suspended in DMEM containing 10% FBS and centrifuged. Pooled cells were plated onto dishes at a density of 10^5 cells/cm² and incubated at 37°C in a humidified air with 5% CO₂ for 72 h, and 0.1 mM bromodeoxyuridine was added into the medium to deplete nonmyocytes. Before the experiment, the cells starved to serum-free DMEM for another 24 h, and then treated with various agents. Neonatal cardiomyocytes culture under a condition of 2% O₂ ~ 95% N₂ for 24 h to mimic ischemia *in vivo*. Tanshinone IIA and SB203580 were given 30 min precede hypoxia.

Fig. 1. The Chemical Structure of Tanshinone IIA.

Parameters	LVSP mmHg	LVEDP mmHg	+dp/dt mmHg/s	-dp/dt mmHg/s	n
Control	118.9±2.3	4.7±0.3	3443±30	-3412±80	8
MI	79.8±2.4*	12.9±0.8*	2040±160*	-2428±40*	6
Tan	104.0±1.0 ⁺	7.1±0.2 ⁺	3362±60 ⁺	-3038±100 ⁺	6

Table 1. The cardiac functional parameters. Effects of Tanshinone IIA on Hemodynamic Parameters during Coronary Artery Occlusion. LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; dp/dt: time derivatives of pressure. MI: myocardial infarction; Tan: Tanshinone IIA (20 mg/kg/d); Values are means±SEM. * $p < 0.05$ vs. control group, ⁺ $p < 0.05$ vs. MI group.

Fig. 2. Effects of Tanshinone IIA on miR-1 and Cx43 Protein in Infarct Hearts. Cardiac miR-1 level was significantly increased and the density of Cx43 was reduced in MI hearts. In contrast, the level of miR-1 and the density of Cx43 returned toward baseline values after tanshinone IIA treatment. (A) Real-time PCR shows the miR-1 levels of MI hearts with or without Tan treatment (B) Western blot analyzes Cx43 protein, * $p < 0.05$ vs. control group; ⁺ $p < 0.05$ vs. MI group, $n = 6$.

Data Analysis

All data are presented as mean ± SEM. There are more than two groups of continuous variables in this study, so one-way analysis of variance ANOVA followed by Bonferroni or Dunnett's post-hoc test was used for multiple comparisons. A two-tailed value of $p < 0.05$ is considered statistically significant difference. Data are analyzed using the GraphPad Prism 5.0 and SPSS 14.0.

Results

Tanshinone IIA improves the hemodynamic parameters of MI rat hearts

After 3 months ligation of the anterior descending coronary artery to create chronic MI, significant reduction of cardiac LVSP and increase in LVSDP were observed (Table 1). The values of +dp/dt and -dp/dt were also markedly diminished, indicating an impairment of the cardiac contractility. The deterioration of cardiac dysfunction induced by chronic MI was significantly improved by administration of tanshinone IIA (20 mg/kg/d, intragastrically) for 3 months. LVSP markedly

increased, and LVSDP significantly decreased, and the decreases in ± dp/dt seen with the infarcted rats were resumed by tanshinone IIA (Table 1).

Tanshinone IIA depresses miR-1 level in MI rat myocytes

Consistent with our previous studies, miR-1 expression was remarkably increased (3.7-fold) in the heart of MI rats compared to control, which resulted in generation of ventricular arrhythmias[1, 10]. Administration of tanshinone IIA diminished the up-regulated miR-1 level after 7 days pretreatment and 3-month application as shown in Figure 2A. Moreover, tanshinone IIA was able to rescue the diminished protein level of Cx43, a target of miR-1 as revealed by Western blot analysis (Fig. 2B).

Tanshinone IIA regulates p38 MAPK pathway in MI rat myocytes

P38 MAPK is known to transducer apoptotic or death signal in cardiomyocytes, and many disease and stress conditions such as ischemia result in the activation

Fig. 3. Effects of Tanshinone IIA on Ischemia-induced p38 MAPK Phosphorylation in Cardiomyocytes. Rats were subjected to ligating or treated with Tan (20 mg/kg/d) for 3 months, and (A) the phosphorylation level of p38 MAPK was determined by Western blot analysis, and p38 MAPK protein values were measured in parallel. (B) SRF and (C) MEF2, as downstream transcription factors were determined, as well. The protein density was standardized by GAPDH. * $p < 0.05$ vs. control group, $^+p < 0.05$ vs. MI group, $n = 5$.

Fig. 4. Tanshinone IIA Protects Cardiac Myocytes from Hypoxic Injury. Cardiac myocytes were incubated in 2% O₂~95% N₂ for 24 h in the presence or absence of 10 μ M Tan, as indicated. The levels of p-p38 MAPK (A) and SRF (C) and MEF2 (D) and Cx43 (E) were determined by Western blot analysis. Total p38 MAPK protein values were measured in parallel. Meanwhile, the effect of Tan on miR-1 level was also examined using Real-time PCR (B). Results are expressed by normalizing to 100% for vehicle-treated samples. Data are plotted as the mean ($\pm$ SEM). * $p < 0.05$ vs. control cells; $^+p < 0.05$ vs. hypoxia cells, $n = 5$.

and phosphorylation of p38 MAPK pathway [13-14]. Once activated, p38 MAPK translocates into the nucleus, with the participation of a variety of transcription factors,

such as cardiac important transcriptional factors, SRF and MEF2 which promote miR-1 expression, to regulate the expression of genes and cellular response [15].

Fig. 5. The p38 MAPK Inhibitor, SB203580 Protects Neonatal Cardiomyocytes against Hypoxic Injury. Cardiac myocytes were incubated in 2% O₂~95% N₂ or normoxia in the presence of 10 μ M SB203580. SB 203580 was preincubated for 30 min. After 24 h, total RNAs were extracted from cell lysates to measure the miR-1 level (A), SRF (B), MEF2 (C) and Cx43 (D) were quantified with Western blot. Data are plotted as the mean ($\pm$ SEM). * p < 0.05 vs. control cells; + p < 0.05 vs. hypoxia cells, n = 5.

Activation of p38 MAPK (1.8-fold) (Fig. 3A) was consistently observed in our MI rats. Accordingly, SRF and MEF2 were increased by 1.6-fold and 1.7-fold in MI, respectively (Fig. 3B and C). Tanshinone IIA reduced p38 kinases phosphorylation without affecting the total protein levels of the kinases, and then downregulated SRF and MEF2 levels (Fig. 3).

Tanshinone IIA protects culture cells against hypoxic injury

To shed some lights on the potential mechanisms underlying the cytoprotective effects of tanshinone IIA on ischemic myocardium, we measured the effects of tanshinone IIA on hypoxic cardiomyocytes *in vitro*. Under hypoxic condition (95% N₂+2% O₂), activation of p38 MAPK was enhanced by 2.7 folds in cultured myocytes (Fig. 4A). On the other hand, hypoxia induced a 3-fold increase in miR-1 level (Fig. 4B), 1.6-fold and 1.5-fold increase in SRF and MEF2 expression, respectively (Fig. 4C and D), along with decrease in Cx43 protein level (Fig. 4E). All these changes were reversed by tanshinone IIA treatment (Fig. 4).

P38 MAPK inhibitor mediates miR-1 in hypoxic injury myocytes

Treatment of the cultured neonatal cardiomyocytes with SB203580 (10 μ M) to inhibit p38 MAPK activation significantly suppressed miR-1 level (Fig. 5A) and both SRF and MEF2 expression (Fig. 5B and C) in hypoxic myocytes. Meanwhile, the decreased Cx43 under hypoxia was recovered by the inhibitor SB203580 treatment as well (Fig. 5D). These results gave us a clue that p38 MAPK pathway participate in the modulation of tanshinone IIA on miR-1 level in ischemic and hypoxic cardiomyocytes.

Discussion

Myocardial infarction is a major health problem which perplexes human being health. MI always refers to cardiomyocytes ischemia and hypoxia, and even cell death. Infarct usually develops cardiac functional changes and even cardiac sudden death if the heart experiences a sustained ischemic damage. Therefore, it is important for

us to prevent these consequences.

The traditional Chinese herb *Danshen*, derived from the dried root of *Salvia miltiorrhiza* Bunge, was widely used to treatment of cardiovascular diseases [6]. Tanshinone IIA, the most abundant component and structurally representative of the tanshinones and the main lipid-soluble compound in *Danshen*, has been widely reported to reduce the infarct size and improve cardiac function after MI [9]. Recently, our group has demonstrated that tanshinone IIA inhibited the ischemia-induced miR-1 overexpression and protected ischemic hearts from cardiac sudden death [10] as mentioned in the Introduction.

miRNAs are a kind of endogenous noncoding RNAs, about 22 nucleotides in length, that mediate post-transcriptional gene silencing by annealing to inexact complementary sequences in the 3'-untranslated regions of target mRNAs. MiR-1 is skeletal and cardiac muscle specific microRNA necessary for postmitotic muscle growth and differentiation [16-17]. We have shown that miR-1 was overexpressed in individuals with coronary artery disease, and when it was overexpressed in normal or infarcted rat hearts, it exacerbates arrhythmogenesis [1]. Enhanced miR-1 suppressed its relevant targets, GJA1 and KCNJ2 [1].

As many reports have extensively demonstrated that p38 MAPK played an important role in ischemia-induced signaling in mammalian cells, and an increase in phosphorylation of p38 MAPK contributed to myocyte injury [3-4, 14]. In agreement with previous findings, we found that the activation of p38 MAPK was increased following 3 months MI and hypoxic stimulation in cardiomyocytes. Tanshinone IIA decreased p38 MAPK activation in MI rats. Under hypoxic condition, tanshinone IIA deactivated p38 MAPK in cultured myocytes, which was consistent with the results of MI rats. With the special inhibitor, we indicated that SB203580 suppressed p38 MAPK activation downregulated the hypoxia-induced miR-1 increment. What's more, tanshinone IIA and SB203580 reduced the transcription factor SRF and MEF2 levels which could promote the miR-1 expression [10, 18]. This explains the reasons why tanshinone IIA protects cardiac myocytes from ischemic injury.

SRF is an essential cardiac transcription factor, modulating the balance between muscle proliferation and cell migration and proliferation [19-20]. MEF2 is recognized as a muscle-specific DNA binding protein which belongs to MADs family. MEF2 family has four members MEF2A-D, they play a role in growth factor and stress stimulus-induced early gene responses [21-

22]. SRF and MEF2 family members can be targeted by MAP kinases signaling which affects muscle gene expression through modulation of SRF and MEF2 activity [15]. Our results showed that SB203580 markedly inhibited SRF and MEF2 levels. This means that SRF and MEF2 are modulated by p38 MAPK, which is supported by previous observations [23-25]. Therefore, we concerned that activated-p38 MAPK pathway promoted the miR-1 expression in cardiomyocytes with SRF and MEF2, heart special transcription factors mediation. Our study supported a good example for this regulation.

The p38 MAPK subfamily has been identified to consist of four isoforms (α , β , γ and δ), only p38 α and p38 β were expressed in cardiac myocytes [26], whereas p38 MAPK α was proapoptotic, and p38 MAPK β was hypertrophic [27]. P38 α was activated during sustained simulated ischemia, whereas the p38 β isoform was deactivated [4]. MKK3 was predominantly responsible for the activation of p38 MAPK α [28-29]. But some researches identified that MKK3-mediated p38 MAPK activation was not necessary for pathological remodeling in the mouse heart following MI [30], and others have reported that ischemic activation of p38 MAPK was through a TAB1-mediated pathway or autophosphorylation [31-33]. So we suppose that tanshinone IIA may modulate p38 MAPK through some unidentified kinases sensitive to SB203580 or TAB1-associated pathways in our experiment. Dissection of specific signaling events that link tanshinone IIA to suppress p38 MAPK in ischemic and hypoxic cardiac myocytes need further investigation.

Our present results have further revealed that tanshinone IIA protects cardiomyocytes against ischemic and hypoxic injury through inhibiting the upregulated miR-1 in cardiomyocytes. And this cardioprotective effect is associated with the suppression of phosphorylation of p38 MAPK signal pathway in ischemic cardiomyocytes. P38 MAPK is activated once in response to ischemia, and consequently detriments cellular survival. So this study likely provides us a new direction to explore the pathogenesis and therapeutic strategies for clinic disease progression.

Abbreviations

Tan (Tanshinone IIA); SRF (serum reaction factor); MEF2 (myocyte enhancer factor 2); MI (myocardial infarction); LVSP (left ventricular systolic pressure);

LVEDP (left ventricular end-diastolic pressure); $+dp/dt_{\max}$ (maximum rate of pressure rise); $-dp/dt_{\max}$ (maximum rate of pressure decline); I_{to} (transient outward potassium current); LAD (left anterior descending coronary artery); Cx43 (connexin 43); DMEM (Dulbecco's modification of Eagle's medium); DMSO (Dimethyl sulfoxide); FBS (Fetal Bovine Serum); MAPK (mitogen-activated protein kinase); SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole); GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

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