Cryptotanshinone attenuates isoprenaline-induced cardiac fibrosis in mice associated with upregulation and activation of matrix metalloproteinase-2

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Abstract. Cryptotanshinone is an active ingredient of *Salvia miltiorrhiza* that has been used in traditional Chinese medicine for treating cardiovascular disorders. Thus, we investigated the effects of cryptotanshinone on cardiac fibrosis induced by isoprenaline and examined whether cardiac matrix metalloproteinase (MMP)-2 is involved in this process. Male C57BL/6 mice received a daily injection of 0.9% saline, 3 mg/kg isoprenaline or isoprenaline plus 20 mg/kg cryptotanshinone by gastric gavage for 2 weeks. In this study we demonstrated that cryptotanshinone was able to significantly ameliorate isoprenaline-induced cardiac fibrosis, which was associated with a marked upregulation and activation of MMP-2 in the ventricular myocardium. Additionally, we demonstrated that cryptotanshinone dose-dependently upregulated and activated MMP-2 in cultured cardiac fibroblasts. Moreover, incubation with cryptotanshinone also prevented the isoprenaline-induced downregulation and inactivation of MMP-2 in cultured cardiac fibroblasts. Taken together, our data suggest that cryptotanshinone is a novel and potent antifibrotic agent. The present findings further our understanding of the role of MMP-2 in cardiac fibrosis and the antifibrotic mechanisms of cryptotanshinone.

Introduction

Progressive cardiac fibrosis is involved in various processes of cardiovascular diseases. It induces cardiac dysfunction, including myocardial stiffening, and results in reduced myocardial contractility and, ultimately, heart failure (1-3). The attenuation of cardiac fibrosis has been viewed as a novel heart-failure treatment target (4). However, there is currently no effective strategy for the treatment and/or prevention of cardiac fibrosis (5).

The accumulation of extracellular matrix (ECM), which characterizes cardiac fibrosis, is due to the reduced degeneration of collagen (6). The principal enzyme system which is primarily responsible for ECM turnover involves the matrix metalloproteinases (MMPs), specifically MMP-2 (7,8). Indeed, the inappropriate reduction of MMP-2 activity associated with the deposition of collagen has been observed in the development of fibrosis in diabetic cardiomyopathy (9,10). Therefore, therapeutic interventions aimed at the activation of MMP-2 may be considered as one of the most effective strategies for the management of cardiac fibrosis (11).

The Chinese herb *Salvia miltiorrhiza*, which has beneficial effects on the circulatory system, has been widely used in traditional oriental medicine for the treatment of patients with cardiovascular disorders (12,13). In particular, tanshinone, one of the active components of *Salvia miltiorrhiza*, has been reported to have antihypertrophic and antifibrotic effects in cultured cardiac myocytes and cardiac fibroblasts (14,15). Cryptotanshinone, another active compound extracted from *Salvia miltiorrhiza* (16,17), has also been involved in the treatment of cardiovascular diseases. However, there have been no studies performed concerning the possible antifibrotic effect of cryptotanshinone.

The present study aimed to determine whether isoprenaline reduces the protein expression and activity of MMP-2 in cultured cardiac fibroblasts and rat myocardium and whether cryptotanshinone is able to normalize MMP-2 expression and activity and attenuate cardiac fibrosis induced by isoprenaline.

Materials and methods

Animals. Male C57BL/6 mice 6-8 weeks of age were obtained from our local animal center. The mice were housed under a 12-h/12-h day/night cycle, with *ad libitum* food and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the General Hospital of PLA Chengdu Military Area Command (Chengdu, China). The mice were randomly assigned to one of the following groups: i) control (Con, n=8), which received a daily injection of 0.9% saline s.c.; ii) isoprenaline (ISO, n=8), which received 3 mg/kg isoprenaline s.c. once daily; iii) isoprenaline plus

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Table I. Morphological parameters of the mice.

Groups	BW (g)	HW (g)	LVW (g)	HW/BW (g/kg)	LVW/BW (g/kg)
Control	30.5±0.96	0.115±0.003	0.102±0.003	3.76±0.02	3.33±0.04
ISO	31.8±1.04	0.136±0.005[b]	0.121±0.005[b]	4.26±0.03[b]	3.79±0.03[b]
ISO+CTS	31.1±1.04	0.122±0.004[c]	0.110±0.003[c]	3.94±0.03[d]	3.53±0.05[d]

Data are presented as mean ± SEM values of 8 mice per group. BW, body weight; HW, heart weight; LVW, left ventricular weight; HW/BW, heart weight to body weight ratio; LVW/BW, left ventricular weight to body weight ratio; ISO, isoprenaline; CTS, cryptotanshinone. [a]P<0.05, [b]P<0.01 vs. control group; [c]P<0.05, [d]P<0.01 vs. ISO group.

cryptotanshinone (ISO+CTS, n=8), which received 20 mg/kg/day cryptotanshinone (gastric gavage) plus the daily injection of isoprenaline. The amount of cryptotanshinone administered was the same as that in a previous study (18).

Hemodynamic measurement. Following 2 weeks of treatment, all the mice were anesthetized with urethane (1.2 g/kg, i.p.). The right carotid artery was cannulated with a polyethylene tube and forwarded to the left ventricle. The tube was connected to a pressure transducer. The left ventricular pressure was recorded in synchrony with the RM-6000 polygraph system (Nihon Kohden, Tokyo, Japan). The left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP) and left ventricular dP/dt max/min were calculated.

Heart weight. At the end of treatment, the animals were sacrificed under deep anesthesia with urethane and the hearts were removed. The ventricles were blotted and weighed and the whole heart weight/body weight (HW/BW) and left ventricular weight (LVW)/BW were calculated. The left ventricular tissue was dissected and frozen at -70˚C for western blotting and gelatin zymography.

Histological determination of fibrosis. Paraffin-embedded left ventricular tissue was cut into 5-μm sections which were stained with picrosirius red and assessed using standard light microscopy. The correlation between the red-stained and the total area of the whole heart section was analyzed as previously described (19).

Cardiac fibroblast culture. Primary adult mouse cardiac fibroblasts were isolated by proteolytic dissociation of the ventricular tissue and cultured using standard protocols as previously described (20). The ventricles were cut into sections and incubated for 10 min in KB buffer, consisting of 70 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 0.5 mM EGTA, 22 mM glucose, 20 mM taurine, 5 mM creatine, 10 mM succinic acid, 2 mM pyruvic acid, 5 mM ATP, 2 mM butyric acid and 115 U/mg collagenase (pH 7.4). The cell suspension was filtered through a 250-μm nylon mesh and centrifuged at 25 x g for 3 min. The supernatant was centrifuged at 250 x g for 10 min. The cells were resuspended in full growth medium [Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml penicillin G, 100 μg/ml streptomycin and 100 U/ml penicillin]. Four 1 hour later, the nonattached cells were removed. The cells were grown for 4-5 days in full growth medium and observed using light microscopy. The culture contained >95% fibroblasts. Cardiac fibroblasts obtained during early passages were used. One day prior to the experiments, the cells were serum-starved. For one experiment, the fibroblasts were incubated with 0, 3, 10 or 30 μM cryptotanshinone for 24 h. For another experiment, the fibroblasts were treated with vehicle, 10 μM isoprenaline or isoprenaline plus 30 μM cryptotanshinone for 24 h.

Western blotting. Western blot analysis was performed as previously reported (21). Briefly, protein lysates were obtained by homogenizing myocardial tissues or cultured cardiac fibroblasts with lysis buffer containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 20 mM Tris (pH 7.5). The protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein from the heart extracts were separated by SDS-PAGE (12%). The samples were then electroblotted onto a nitrocellulose membrane (Boehringer Mannheim Corporation, Indianapolis, IN, USA) and probed with antibody against MMP-2 (1:500; Sigma-Aldrich Co., St. Louis, MO, USA). After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and bound antibody was visualized using a colored reaction. The relative band densities were quantified by densitometry using the Multi-Analyst software package (Bio-Rad). Equal loading of protein was confirmed by measuring β-actin expression.

Gelatin zymography. The gelatin-degrading activity of MMP-2 in the left ventricular samples and cultured cardiac fibroblasts was identified by zymography using an assay kit (APPLYGEN, Beijing, China), as described previously (22). Briefly, samples were mixed with sample buffer and separated on 7.5% SDS-polyacrylamide gels containing 1 mg/ml of gelatin. Following the removal of SDS, the gel was incubated in buffer overnight and then stained with 0.25% Coomassie brilliant blue G-250. The gelatinolytic activity was detected and quantified using a GS-800 calibrated densitometer (Bio-Rad).

Statistical analysis. Data are presented as the means ± SEM. The comparisons between groups were determined using one-way ANOVA with Student's t-test post hoc test (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result.

Table II. Hemodynamic parameters of the mice.

Groups	LVEDP (mmHg)	LVESP (mmHg)	dP/dt max (mmHg/sec)	dP/dt min (mmHg/sec)
Control	3.2±0.3	86±6	5683±110	4478±88
ISO	6.3±0.4[b]	100±4	5471±110	4153±64[a]
ISO+CTS	5.2±0.3[c]	91±5	5569±75	4306±96

Data are presented as the means ± SEM values of 8 mice per group. LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; dP/dt max, positive-derived pressure; dP/dt min, negative-derived pressure; ISO, isoprenaline; CTS, cryptotanshinone; [a]P<0.05, [b]P<0.01 vs. control group; [c]P<0.05 vs. ISO group.

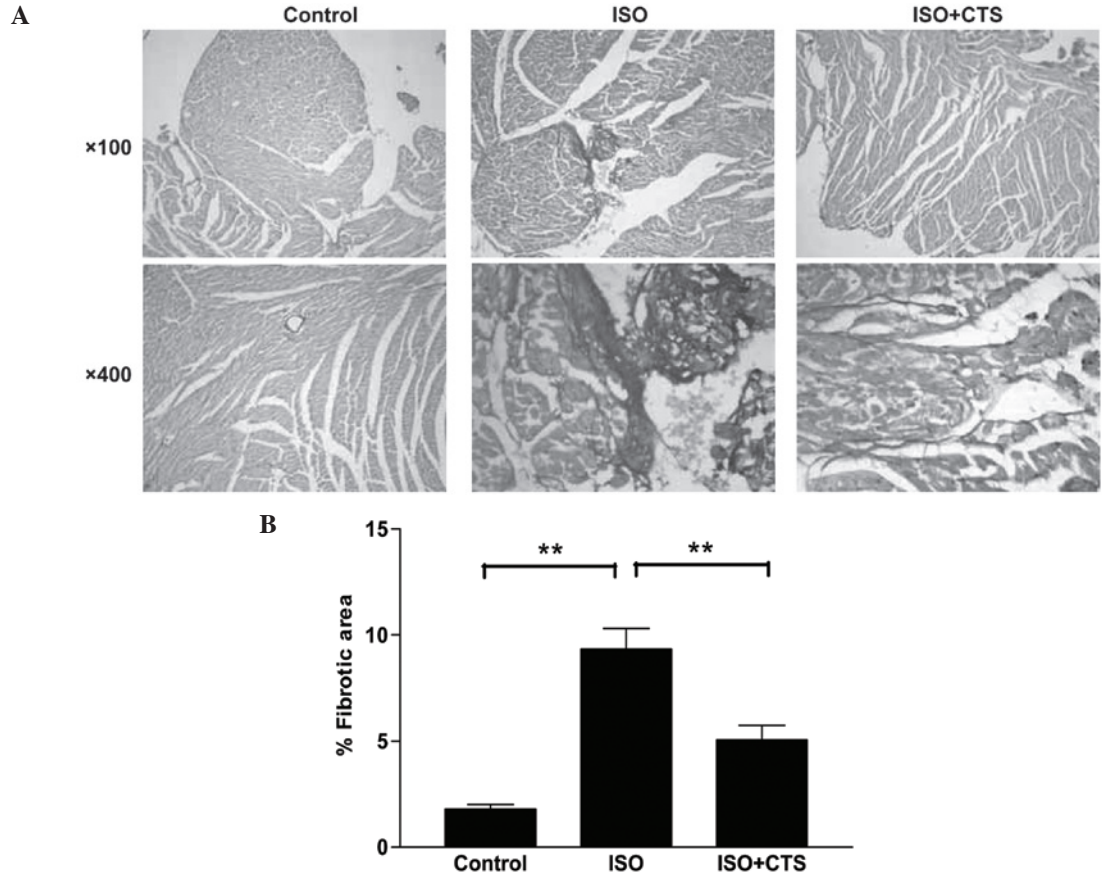

Figure 1. Effect of cryptotanshinone on cardiac fibrosis. (A) Representative photomicrographs of picrosirius red-stained sections of hearts at the level of the papillary muscle; upper panel, original magnification x100; lower panel, original magnification x400. (B) Bar graph showing quantitative analysis of cardiac fibrotic area. ISO, isoprenaline; CTS, cryptotanshinone; data are presented as mean ± SEM; n=5 mice in each group; **P<0.01.

Results

Morphological parameters. The effects of treatment on the morphological parameters of the mice are summarized in Table I. As expected, HW, LVW, HW/BW and LVW/BW were significantly higher in isoprenaline-treated mice compared with the control animals (all P<0.01). Notably, the isoprenaline-induced increases in HW, LVW, HW/BW and LVW/BW were markedly attenuated by cryptotanshinone treatment (P<0.05 or P<0.01).

Hemodynamic parameters. As shown in Table II, isoprenaline treatment elicited a significant increase in the LVEDP (P<0.01) and a marked decrease in the dP/dt min (P<0.05). Notably, the isoprenaline-induced elevation in LVEDP was significantly attenuated by the treatment with cryptotanshinone (P<0.05). By contrast, no significant difference in the LVESP and dP/dt max between the treatment groups was found.

Extent of fibrosis. Representative photomicrographs of the heart are shown in Fig. 1A. The extent of fibrosis was significantly increased in the isoprenaline-treated mice (P<0.01; Fig. 1B). Additionally, this increase was partly, but significantly, prevented by cryptotanshinone treatment (P<0.01; Fig. 1B).

Expression and activity of MMP-2 in ventricular myocardium. Compared with the control group, isoprenaline treatment significantly decreased the protein expression and

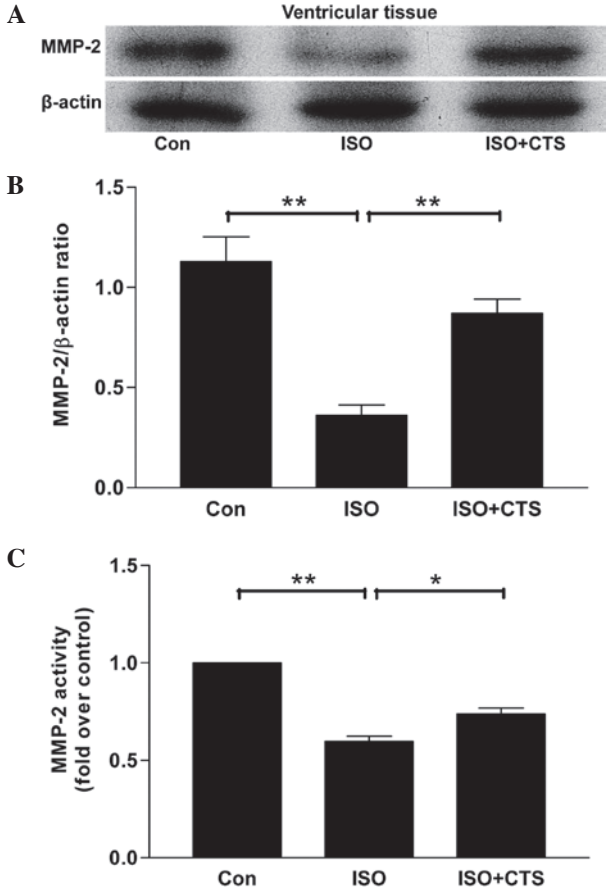

Figure 2. Effects of cryptotanshinone on cardiac MMP-2 protein expression and activity. (A) Representative western blot bands of MMP-2 and β-actin in the ventricular myocardium from control (Con), Isoprenaline-treated (ISO) and isoprenaline plus cryptotanshinone-treated (ISO+CTS) mice. (B) MMP-2/β-actin ratio is shown in the bar graph. (C) Bar graph showing quantitative analysis of MMP-2 activity. Data are presented as mean ± SEM; n=5 mice in each group; *P<0.05; **P<0.01. MMP, matrix metalloproteinase.

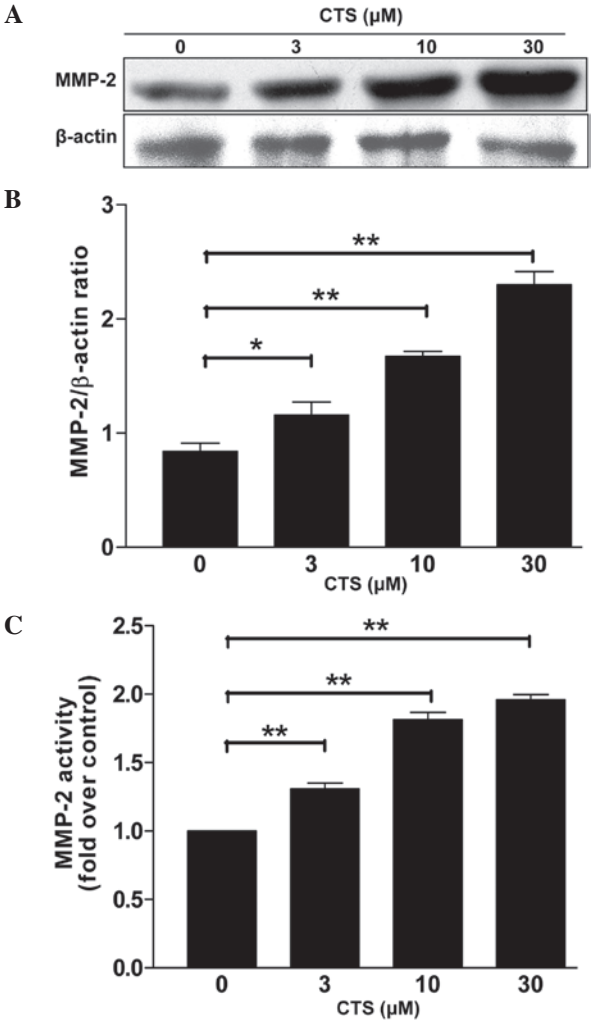

Figure 3. Effects of cryptotanshinone on MMP-2 protein expression and activity in cultured cardiac fibroblasts. (A) Representative western blot bands of MMP-2 and β-actin in cultured cardiac fibroblasts treated with 0, 3, 10 or 30 μM cryptotanshinone (CTS) for 24 h. (B) MMP-2/β-actin ratio is shown in the bar graph. (C) Bar graph showing quantitative analysis of MMP-2 activity. Data are presented as mean ± SEM of three independent experiments; *P<0.05; **P<0.01. MMP, matrix metalloproteinase.

degenerative activity of MMP-2 (P<0.01; Fig. 2). Moreover, the inhibitory effects of isoprenaline were markedly attenuated by cryptotanshinone treatment (P<0.01 or P<0.05; Fig. 2).

Expression and activity of MMP-2 in cultured cardiac fibroblasts. In the cultured cardiac fibroblasts, isoprenaline incubation dose-dependently increased the protein expression and degenerative activity of MMP-2 (P<0.01 or P<0.05; Fig. 3). Furthermore, the protein expression and degenerative activity of MMP-2 were significantly reduced in the isoprenaline-treated fibroblasts (both P<0.01, Fig. 4) but these decreases were partly prevented by treatment with cryptotanshinone (both P<0.05, Fig. 4).

Discussion

There are two novel findings in the present study, which used *in vitro* cultured cardiac fibroblasts and an *in vivo* mouse model of isoprenaline-induced cardiac fibrosis. First, cryptotanshinone was able to partly, but significantly, prevent the cardiac fibrosis caused by isoprenaline injection. Second, the antifibrotic effect of cryptotanshinone was associated with comparable elevations of MMP-2 protein expression and activity. These findings suggest that the downregulation and inactivation of MMP-2 contributes to the isoprenaline-induced cardiac fibrosis and cryptotanshinone-induced attenuation of cardiac fibrosis.

MMP-2, which has collagenolytic activity on types I, II and III collagen, has been implicated in cardiac fibrosis (23). Several lines of evidence have indicated that impaired matrix degradation is associated with reduced MMP-2 activity (9,10,24). However, the results were inconsistent and appeared to depend on the animal model used. Chronic pressure overload in MMP-2-deficient mice is associated with reduced cardiac interstitial fibrosis and MMP-2 transgenic mice have been reported to be associated with increased replacement fibrosis and perivascular fibrosis (25,26). The results of the present study support the theory that the degenerative activity of MMP-2 is markedly impaired in the fibrotic myocardium. Moreover, this phenomenon was also observed in cultured cardiac fibroblasts stimulated with isoprenaline

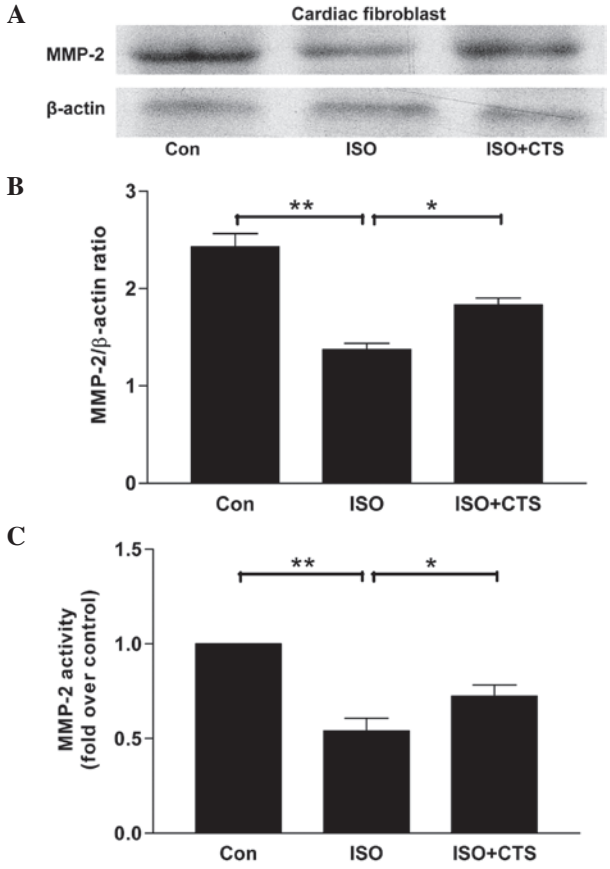

Figure 4. Effects of cryptotanshinone on isoprenaline-induced downregulation and inactivation of MMP-2 in cultured cardiac fibroblasts. (A) Representative western blot bands of MMP-2 and β-actin in cultured cardiac fibroblasts treated with vehicle (Con), isoprenaline (ISO) (10 μM) or isoprenaline plus cryptotanshinone (ISO+CTS) (30 μM) for 24 h. (B) MMP-2/β-actin ratio is shown in the bar graph. (C) Bar graph showing quantitative analysis of MMP-2 activity. Data are presented as mean ± SEM of three independent experiments; *P<0.05; **P<0.01. MMP, matrix metalloproteinase.

in the present study. Similarly, stimulation with high glucose has also been found to decrease the activity of MMP-2 in rat mesangial cells (27). Collectively, the augmentation of MMP-2 activation may be a useful therapeutic strategy to prevent cardiac fibrosis.

Salvia miltiorrhiza is a well-known herb used in traditional Chinese medicine for the treatment of cardiocerebrovascular diseases. The two main active ingredients extracted from the *Salvia miltiorrhiza* root are tanshinone and cryptotanshinone, which have distinct biological effects. Although tanshinone has been shown to have an antifibrotic effect in cultured cardiac fibroblasts (14), the role of cryptotanshinone in the development of cardiac fibrosis has not been reported. The present study demonstrated for the first time that treatment with cryptotanshinone is an effective method to prevent isoprenaline-induced cardiac fibrosis. Moreover, our results also suggest that the antifibrotic effect of cryptotanshinone is due to the upregulation and activation of MMP-2. However, other mechanisms cannot be excluded. A previous study demonstrated that cryptotanshinone may inhibit proinflammatory cytokine production, reduce neutrophil infiltration and downregulate adhesion molecules through the inhibition of NF-κB-activation during ischemia and reperfusion and finally attenuate ischemia and reperfusion-induced microcirculatory disturbances (28). Therefore, anti-inflammatory action may also be involved in cryptotanshinone-induced cardiac protection.

The inhibitory effect of cryptotanshinone in isoprenaline-induced increases in HW, LVW, HW/BW and LVW/BW indicated that cryptotanshinone may have antihypertrophic action. The beneficial effect in hemodynamic homeostasis further confirmed this hypothesis. However, this issue warrants further investigation.

In summary, our data reveal that cryptotanshinone is able to upregulate and activate MMP-2 in cultured cardiac fibroblasts and that this effect is able to reverse the isoprenaline-induced downregulation and inactivation of MMP-2 in cardiac fibroblasts. Our data also reveal that the antifibrotic effect of cryptotanshinone is associated with the upregulation and activation of MMP-2 in the ventricular myocardium. Taken together, our findings further support the hypothesis that MMP-2 activation has therapeutic potential against the development of cardiac fibrosis and highlight the mechanisms involved in the antifibrotic effect of cryptotanshinone.

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