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Salutary effects of *Corydalis yanhusuo* extract on cardiac hypertrophy due to pressure overload in rats

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Abstract

We have evaluated the effects of an alcohol extract from the rhizome of *Corydalis yanhusuo* W.T. (CY), a well-known traditional Chinese medicinal herb, on pressure-overloaded cardiac hypertrophy induced by transverse abdominal aorta constriction (TAAC) in rats. Rats were given vehicle or CY extract (200 or 50 mg kg^{-1} per day) from the second week after induction of pressure overload, for a period of 7 weeks. Haemodynamic parameters, relative heart weight and myocyte cross-sectional area were measured in each group. We also estimated left ventricular (LV) collagen volume fraction (CVF) using Masson trichrome staining, and type I collagen expression by Western blot assay. Chronic TAAC caused notable cardiac hypertrophy and heart dysfunction. Significant collagen deposition and greater type I collagen expression were found in model control rats. These changes were not significantly reversed after treatment with 50 mgkg^{-1} CY, whereas 200 mgkg^{-1} significantly improved heart function and prevented cardiac hypertrophy, with parallel reductions in myocardial fibrosis, as evidenced by reduced LV CVF and reduced levels of type I collagen. In conclusion, chronic treatment of rats with CY extract attenuated development of cardiac hypertrophy.

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Introduction

Cardiac hypertrophy is a growing public health problem worldwide, due in part to the aging population (Hilfiker-Kleiner et al 2006). Cardiac pressure overload, one of the main causes of cardiac hypertrophy, occurs in many clinical settings, including hypertension, and mitral and aortic valve stenosis. When subject to one of these negative stimuli, the heart undergoes cardiac hypertrophy in order to adapt to the increase in pressure on the cardiac tissue. The hypertrophic growth of cardiac myocytes is paralleled by progressive hyperplasia of fibroblasts and the accumulation of extracellular matrix components, including collagens, which leads to cardiac fibrosis (Balke & Shorofsky 1998). Recent studies have highlighted the importance of cardiac fibrosis in the pathogenesis of cardiac hypertrophy, which has been found to detrimentally affect the functional properties of the heart by increasing diastolic stiffness, impairing systolic function, causing myocardial heterogeneity and favoring re-entry arrhythmias (Nadal-Ginard et al 2003; Carreno et al 2006; Kerkela & Force 2006). Cardiac fibrosis may therefore be a determinant of cardiac hypertrophy.

In recent years, medicinal herbs and their extracts have received great attention for salutary effects on cardiac hypertrophy (Valli & Giardina 2002; Rizik et al 2006; Yeh et al 2006). *Corydalis yanhusuo* W.T. (CY), a well-known traditional Chinese medicinal herb, is widely used as an analgesic in angina pectoris and other cardiovascular disorders. Previous studies have shown that extract or principles from CY could possess various cardiovascular activities (Jin 1987), such as antihypertensive activity (Chueh et al 1995a, b; Xing et al 1994; Lin et al 1996) and anti-arrhythmic action (Wang & Li 1987; Zeng et al 2000a) and improves cardiac haemodynamics and function (Liu & Zhao 1987; Xuan et al 1992). We have also demonstrated that CY extract exerted beneficial effects on heart subjected to myocardial ischaemia/reperfusion or myocardial infarction (Ling et al 2006). Although the salutary effects on the cardiovascular system have been described for CY extract or *DL*-tetrahydropalmatine (*DL*-THP), a principal active component of CY, the possible beneficial effects of CY on cardiac hypertrophy due to chronic pressure overload is unknown. In the current research, we used a reproducible cardiac hypertrophy model – transverse abdominal aorta constriction (TAAC) – in rats to investigate the cardioprotective effects of CY on

cardiac hypertrophy and fibrosis, and haemodynamic dysfunction.

Materials and Methods

Plant material and extraction

The rhizome of CY W.T., collected from Pan'an country of Zhejiang Province, was supplied by Xi'an Sanjiang Bio-Engineering Co. Ltd (Xi'an, PR China). The species of medicinal herb were identified by Professor Jinxiang Yang (Northwest Institute of Botany, Chinese Academy of Sciences Xi'an, PR China). Voucher specimens (250314) were deposited at the Herbarium, Laboratory of Pharmacology on Traditional Chinese Medicine of Zhejiang University, PR China.

The extract was separated and analysed as described previously (Wu et al 2007). The CY rhizomes was cut into small pieces, powdered and then extracted three times using 85% alcohol. After retrieving the alcohol, the extract was freeze-dried, giving a powdery crude extract of CY. Further chemical analysis was performed using a Waters 2695 HPLC system (Milford, MA, USA). The analytical column was a Zorbax SB-C18 (5 μm, 4.6×250 mm) with a Zorbax SB-C18 (5 μm, 4.6×45 mm) guard column (both from Agilent Technologies, Shanghai, PR China). The eluent was 70% 3.7 mM phosphoric acid buffer (pH 2.55) and 30% acetonitrile (Fu et al 1986a, b; Ou et al 2006; Yuan et al 1996).

Animals

Male Sprague–Dawley rats (200±20 g body weight) were provided by Laboratory Animal Center of Zhejiang University. Animals were housed in Makrolon cages (five rats per cage) under controlled conditions of constant temperature and humidity and exposed to a 12 h dark–light cycle. Rats had free access to a standard diet and drinking water. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Zhejiang University.

Induction of heart failure

Cardiac hypertrophy was induced by TAAC. Briefly, rats underwent laparotomy under sodium pentobarbital (45 mg kg^{-1} i.p.) anaesthesia to expose the suprarenal abdominal aorta. A blunt 22-gauge needle was placed adjacent to the aorta and a ligature (5-0 silk) was tied snugly around the aorta and needle. The needle was then removed, leaving the internal diameter of the aorta approximately equal to that of the needle. Successful bands were snug while maintaining blood flow to the kidneys and lower extremities. Sham-operated animals underwent the same surgical procedure with an untied ligature placed in the same location. Mortality within 1 week of the operation was approximately 10%.

Experimental protocol

One week after the operation, TAAC rats were divided into three groups according to oral drug treatment: the control group (n=6) were given vehicle (physiological saline) 10 mL kg^{-1}; treatment groups were given CY extract 200 mg kg^{-1} (n=6) or 50 mg kg^{-1} (n=6). The fourth group comprised the sham-operated rats (n=6), which received physiological saline (10 mL kg^{-1}). Treatments were administrated once a day for seven consecutive weeks.

Measurement of haemodynamic parameters

Seven weeks after CY treatment, animals were anaesthetized with sodium pentobarbital (45 mg kg^{-1} i.p.). A catheter was inserted into the abdominal aorta via the femoral artery to measure arterial blood pressure. The right carotid artery was cannulated and a catheter inserted via the carotid artery into the left ventricle. When a stable and reproducible pressure reading was obtained, left ventricular systolic pressure (LVSP), LV end-diastolic pressure (LVEDP) and rate of rise and decline of LV pressure (+/− dp/dtmax) were measured via a pressure transducer interfaced to a recorder (Biopac Systems Inc., Aero Camino Goleta, CA, USA).

Measurement of relative heart weight

Hearts were removed and weighed (giving the wet weight). Relative heart weight, calculated by dividing the heart weight (HW) by body weight (BW), was used as a measurement of cardiac hypertrophy.

Measurements of left ventricular collagen volume fraction

Myocardium was separated from LV segments and fixed in 10% formalin. Masson trichrome staining was used to determine the collagen volume fraction (CVF). Briefly, serial 5 μm sections were cut from paraffin-embedded myocardium, then deparaffinized and hydrated. Slides were fixed in Bouin's solution for 1 h in a 60°C water bath, then cooled, washed under running tap water for 5 min and rinsed with distilled water. Slides were then immersed in working haematoxylin solution for 10 min. After being incubated with ponceau and acid fuchsin solution for 5 min, slides were transferred to phosphomolybdic acid solution and aniline blue solution for 5 min each and then rinsed. Finally, slides were placed in aqueous acetic acid solution for 1 min, dehydrated, cleared and covered. As a result, collagen was stained blue and cardiac myocytes red. To detect extracellular collagen deposition, 10 random fields of each stained section were analysed by an examiner who was blinded to the animals' treatment, using an Image C Morphology Analysis System (Chansan Instrument Co., Shanghai, China). CVF was calculated as the mean ratio of connective tissue to the total tissue area of all measurements of the section, omitting fibrosis of the perivascular, epi- and endocardial areas.

Cardiomyocyte cross-sectional area

H&E-stained tissue sections from three hearts in each treatment group were studied at 200× magnification. In each field, the cross-sectional area of all transversally cut myocytes showing a nucleus was measured (Image C Morphology

Analysis System). Six to eight randomly selected fields spanning the septum, apex and free wall were studied per tissue section. Cell borders were planimetered manually by an operator who was blinded to treatment. At least 50 myocytes in each region were measured.

Detection of type I collagen expression by Western blot assay

Tissues for Western blot analysis were taken from LV segments in each group, pulverized in liquid nitrogen and homogenized in lysis buffer containing 3% Triton X-100, 300 mM NaCl, 100 mM Tris (pH 7.3), 1 mM Na_3VO_4, 20 mM EDTA, 1% Nonidet P-40 (NP-40), 2 mM phenylmethyl sulfonylfluoride (PMSF) and protease inhibitors (aprotinin, pepstatin and leupeptin, all 1 μg mL^{-1}). The homogenate was centrifuged at 14000 rpm for 15 min at 4°C and the resulting supernatant collected. After determining protein concentration using a commercial assay (DC protein assay; BioRad, Hercules, CA, USA), 50 μg total protein was boiled for 5 min in loading buffer containing 0.25 M Tris (pH 6.8), 20% glycerol, 4% SDS and 0.05% bromophenol blue, and loaded onto SDS-PAGE. The proteins in the gels were transferred electrophoretically to polyvinylidene difluoride sheets for 90 min at 2 mA cm^{-1}. The sheets were blocked in Tris-buffered saline (TBS) containing 5% skimmed milk and 0.1% Tween-20 (TBS/T) for 1 h and subsequently exposed to rabbit polyclonal anti-rat collagen I (1:800; Boster, Wuhan, PR China) overnight at 4°C in a buffer containing 10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20 and 5% skimmed milk. Bound antibody was detected by horseradish-peroxidase-conjugated anti-rabbit IgG (1:2000) for 1 h at room temperature. After extensive washing with TBS/T, enhanced chemiluminescence detection reagents were employed and the blots were exposed to Kodak X-0mat BT film. Digital images of films were captured and quantified using a bio-imaging system (BioRad).

Statistical analysis

Data are means ± s.d. Differences between groups were assessed by one-way analysis of variance followed by Dunnett's two-sided comparison test. A P value less than 0.05 was considered significant.

Results

Chemical composition of CY extract

A lot of alkaloids were identified from the CY rhizome extract, including THP, palmatine, dehydrocorydaline (DHC), protopine and other alkaloids (Figure 1). The extract contained 15.70% DL-THP and 1.28% DHC, as determined by HPLC.

Effect of CY extract on cardiac hypertrophy

Eight weeks' TAAC caused marked cardiac hypertrophy in the vehicle control rats, indicated by increased HW/BW ratio

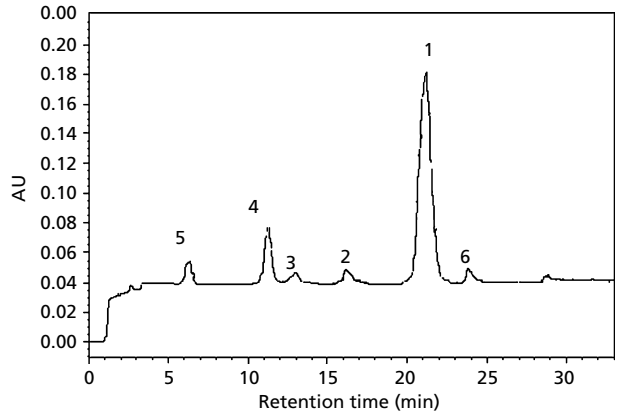

Figure 1 Typical HPLC chromatogram of the extract from *Corydalis yanhusuo*. Peak 1 = tetrahydropalmatine; peak 2 = dehydrocorydaline; peak 3 = palmatine; peak 6 = protopine; Peaks 4 and 5 were unknown constituents.

(4.13 ± 0.33 vs 3.06 ± 0.17 mg g^{-1} in sham-operated rats, $P < 0.01$), which was significantly reduced by CY 200 mg kg^{-1} (3.49 ± 0.26 mg g^{-1}, $P < 0.05$ vs cardiac hypertrophy control group). TAAC induced LV hypertrophy at the cellular level, confirmed by a significantly increased myocyte cross-sectional area (557.8 ± 77.6 μm^2 in vehicle rats vs 213.1 ± 16.7 μm^2 in sham-operated rats, $P < 0.01$), and this was significantly reduced by CY 200 mg kg^{-1} (373.2 ± 82.1 μm^2, $P < 0.05$ vs vehicle-treated controls).

Effect of CY extract on haemodynamic parameters

Haemodynamic measurements revealed cardiac pressure overload and substantial heart dysfunction, as evidenced by increased LVEDP, decreased +/– dp/dtmax and cardiac hypertrophy in control rats. Heart rate was significantly decreased in CY-treated hearts but CY had no apparent effect on mean arterial blood pressure. CY 200 mg kg^{-1} significantly inhibited the deterioration of these haemodynamic parameters compared with vehicle control rats (Table 1).

Effect of CY extract on collagen deposition and collagen volume fraction

Marked collagen deposition (detected by Masson trichrome staining) was found in vehicle control hearts (Figure 2A), confirmed by a raised CVF (8.69 ± 1.62% in vehicle control rats vs 2.79 ± 0.98% in sham rats, $P < 0.01$; Figure 3). CY 200 mg kg^{-1} prevented collagen accumulation (Figure 2C) and reduced CVF (5.39 ± 1.42% $P < 0.01$ vs vehicle control rats).

Effect of CY extract on type I collagen expression

Type I collagen is predominant in the cardiac interstitium. Western blot assay (Figure 4) showed that type I collagen expression was markedly increased in vehicle control compared with sham-operated rats. Treatment with CY 200 mg kg^{-1}

Table 1 Effect of *Corydalis yanhusuo* (CY) extract on haemodynamic parameters

	Sham	Vehicle	CY 50 mg kg^{-1}	CY 200 mg kg^{-1}
Heart rate (beats/min)	398 ± 12	530 ± 18†	450 ± 15	440 ± 13*
Mean arterial pressure (mmHg)	131 ± 5	145 ± 11	137 ± 7	132 ± 7
LV systolic pressure (mmHg)	145 ± 9	140 ± 6	137 ± 8	136 ± 7
LV end-diastolic pressure (mmHg)	3 ± 1	17 ± 4†	16 ± 3	12 ± 3*
+ dp/dtmax (mmHg s^{-1})	10134 ± 794	6312 ± 691†	7294 ± 623	8411 ± 561**
– dp/dtmax (mmHg s^{-1})	7258 ± 578	4454 ± 498†	4628 ± 564	5821 ± 770*

LV, left ventricular; +/– dp/dtmax, rate of rise/fall in LV pressure. Data are means ± s.d., n = 6 rats. †$P < 0.01$ vs sham-operated rats; *$P < 0.05$; **$P < 0.01$ vs vehicle.

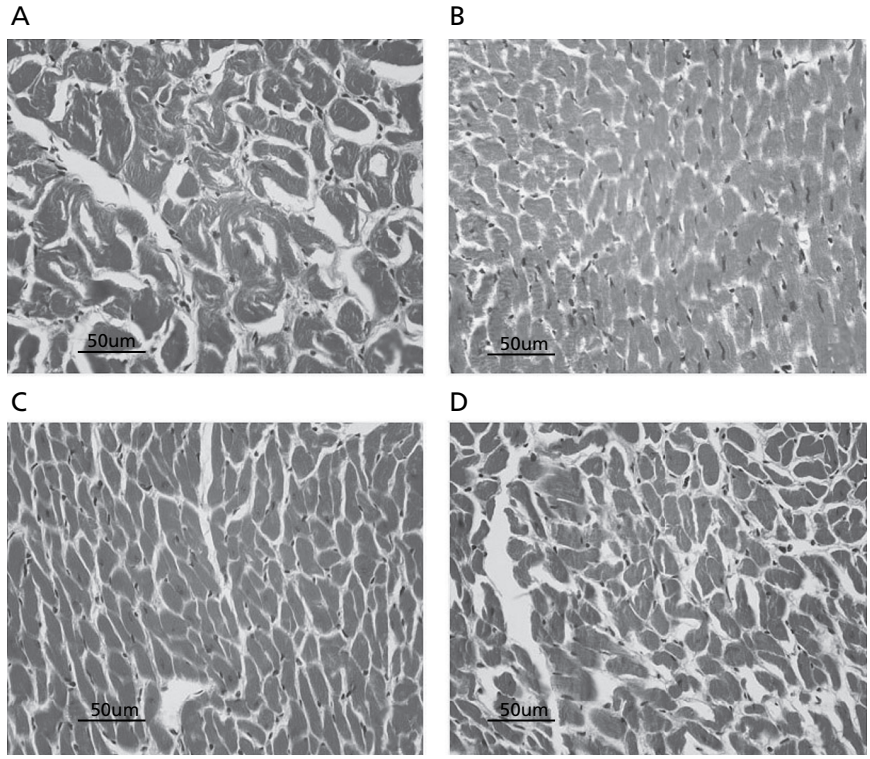

Figure 2 Detection of collagen deposition by Masson trichrome staining in vehicle controls (A), sham-operated rats (B) and *Corydalis yanhusuo* (CY) 200 mg kg^{-1} (C) and 50 mg kg^{-1} groups (D). Interstitial collagen is stained blue and cardiomyocytes red. Eight weeks' abdominal aorta stenosis caused marked collagen accumulation, which was decreased by CY in a dose-dependent manner.

was associated with a significant reduction in collagen I levels, implying the normalization of the extracellular matrix (ECM) composition.

Discussion

The prevalence of cardiac hypertrophy has reached epidemic proportions worldwide (Balke & Shorofsky 1998; Hilfiker-Kleiner et al 2006; Liu et al 2006). This excessive prevalence is magnified further by low success rates in the treatment of hypertension in order to reverse cardiac hypertrophy (Carreno et al 2006; Heineke & Molkentin 2006). These findings have interesting implications for treatment strategies using traditional Chinese herbs that aim to prevent and reverse cardiac hypertrophy.

Myocardial hypertrophy is a response of cardiac muscle to altered conditions caused by a large number of physiological and pathological stimuli and is a major predictor of progressive heart disease. After 8 weeks of pressure overload, rats with pronounced cardiac hypertrophy showed significant decreases in LVSP and +/– dp/dtmax, whereas LVEDP was markedly increased. Pathological hypertrophy is also associated with impaired myocardial vascularization, unfavourable

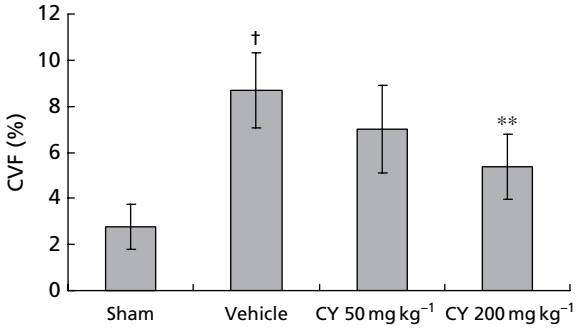

Figure 3 Effect of *Corydalis yanhusuo* on collagen volume fraction (CVF), calculated as the mean ratio of connective tissue to the total tissue area of all measurements of the Masson-trichrome-stained sections, omitting fibrosis of the perivascular, epi- and endocardial areas. Data are means ± s.d., n = 6 rats. $^{**}P < 0.01$ vs vehicle group; $\dagger P < 0.01$ vs sham group.

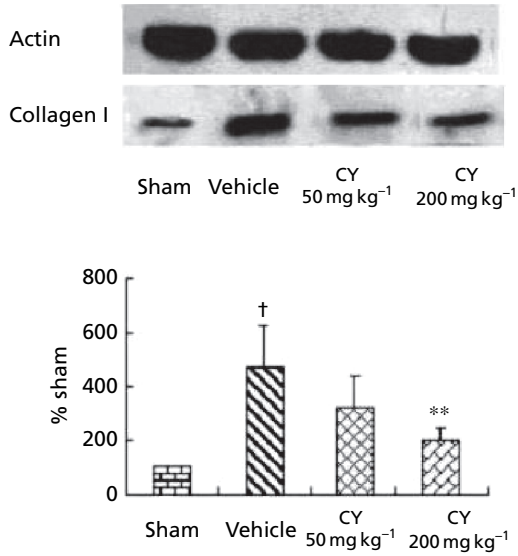

Figure 4 Expression of type I collagen protein visualized by Western blot assay (A) and type I collagen protein levels in control and *Corydalis yanhusuo* (CY)-treated rats as a percentage of that in sham-operated rats. Protein levels were calculated by densitometry. Data are means ± s.d., n = 6 rats. $\dagger P < 0.01$ vs sham group; $^{**}P < 0.01$ vs vehicle controls.

changes in the ECM composition and fibrosis. These results are consistent with other reports showing abnormalities in cardiac performance (Nadal-Ginard et al 2003; Liu et al 2006). At the lower dose (50 mg kg^{-1} per day) CY showed only slight effects on the abnormalities of hypertrophied heart. The pressure-overload-induced changes in heart function, cardiomyocte size, collagen deposition and expression of type I collgen were greatly attenuated by 200mg kg^{-1} CY (equivalent to 2 g dried medicinal herbs, according to the 2005 version of the Pharmacopeia of PR China).

In contrast to previous studies, which mainly examined acute and chronic effects in myocardial infarction (Zeng et al 2000a; Ling et al 2006), our results demonstrate that chronic treatment with CY extract can improve cardiac function and structure in a rat model of cardiac hypertrophy due to pressure overload.

Over the last decade a multitude of extracellular factors and signalling pathways have been shown to be involved in cardiac hypertrophy, such as the sympathetic nervous system, the renin–angiotensin system, reactive oxygen species, apoptosis, calcium ion handling, and so on. The CY extract contained approximately 10 alkaloids including DHC, corydaline, *DL*-THP, protopine, tetrahydrocoptisine, tetrahydrocolumbamine and corybulbine, so these might exert a variety of cardioprotective effects through various mechanisms.

There is substantial evidence to support the notion that calcium signalling pathways contribute to the progression of cardiac hypertrophy. High calcium concentrations within cardiomyocytes cause an increase in protein synthesis, transcriptional activation of immediate early genes and induction of autocrine/paracrine growth factors, leading to an increase in ventricular mass. There is evidence that calcium channels play an important role in the activation of G-protein-coupled and epidermal growth factor receptors in cardiomyocytes, and produce cardiac hypertrophy. Calcium channel blockers are widely used for the treatment of hypertrophy (Balke & Shorofsky 1998; Schwarz et al 2003; Carreno et al 2006; Kerkela & Force 2006). Previous studies have reported that some constituents of the CY extract interfere with cardiomyocyte calcium channels. THP, one of the main active principles, has been demonstrated to be a potent calcium channel blocker (Xu et al 1996), and decreased calcium ion concentrations in ventricular myocytes in a dose- and frequency-dependent manner (Chan et al 1999). *L*-THP inhibited calcium ion overload in cultured rat cardiomyocytes during hypoxia and reoxygenation (Zeng et al 2000b) and had a moderate inhibitory effect on L-channel calcium currents (Huang et al 1999). CY also exerted analgesic activity through inhibition of calcium channels (Hu et al 1994). Inhibition of calcium channels may therefore represent one of the mechanisms by which CY exerts anti-hypertrophic action.

Release of norepinephrine from the sympathetic nerve terminal in the long term is a potent stimulus for cellular growth via adrenergic mechanisms (Osadchii 2007). Previous studies have shown that catecholamines are direct mediators of hypertrophy in rat myocytes and of proliferation and collagen production in fibroblasts (Oliveira & Krieger 2005; Wang et al 2005). Thus, increased cardiac sympathetic nervous outflow seems to be a critical factor in the pathophysiological process that leads from compensated cardiac hypertrophy to cardiac failure. Previous studies have demonstrated that norepinephrine concentrations in the heart, aorta and femoral artery are markedly reduced by THP, a constituent of CY (Xu et al 1987; Xing et al 1994). THP also significantly decreased the concentrations of norepinephrine and dopamine in the cortex and brain stem, and induced hypotension and bradycardia in rats (Hsieh et al 1994; Chueh et al 1995a; Lin et al 1996; Chang & Lin 2001). Therefore, the modulation of cardiac hypertrophy by CY may involve both direct adrenoceptor stimulation in cardiac cells and secondary effects on haemodynamics.

Previous studies have reported that pathological LV hypertrophy caused by haemodynamic load is associated with abnormal accumulation of fibrillar collagen within the extracellular space. Perivascular fibrosis leads to myocardial ischaemia and ventricular stiffness (Cantor et al 2005; van den Bosch et al 2006). Cardiac fibrosis is characterized by extensive perivascular and interstitial accumulations of fibrous tissue, which is largely composed of a complex network of fibrillar collagen. Interstitial collagen restrains the shortening of cardiac cells or reduces force transmission and cell-to-cell mechanical coupling. Its disproportional accumulation has therefore been implicated as a major determinant of impaired stiffness and pumping capacity, and excessive accumulation may account for ventricular dysfunction. The current study revealed that 8 weeks' TAAC caused a marked haemodynamic compromise, as evidenced by significant elevation in LVEDP and decrease in +/− dp/dtmax. One explanation for the deterioration in LV function is an increase in interstitial collagen, indicated by raised CVF. CY therapy reduced cardiac fibrosis and collagen deposition, accompanied by recovery from cardiac dysfunction. This study provides the first evidence that CY decreases collagen deposition and reduces type I collagen expression, accompanied by the prevention of cardiac hypertrophy and improvement in heart function in rats with cardiac hypertrophy induced by TAAC. It is possible that the attenuation of collagen deposition may be due to a decrease in mRNA levels.

Some alkaloids of CY, such as THP, have been reported to exhibit potent antioxidative activity in both lipid peroxidation and haemolysis assays (Ng et al 2000). It is evident that CY exerts beneficial effects via several mechanisms. Although the results of this study are partly supported by these explanations, information on the actions of CY in cardiac hypertrophy due to chronic pressure overload is still scant at present. Whether the improved cardiac function observed with CY is due to these mechanisms remains elusive, and these aspects need further investigation.

Conclusion

The salutary effects of CY therapy on pressure-overloaded cardiac hypertrophy were associated with the inhibition of heart hypertrophy, reduction in cardiac fibrosis and improvement in LV function. These data support the theory that CY extracts target pathological aspects of the hypertrophic process.

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