JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 1687–1694

Received March 29, 2008
Accepted August 18, 2008
DOI 10.1211/jpp/60.12.0016
ISSN 0022-3573

Total triterpene acids, active ingredients from Fructus Corni, attenuate diabetic cardiomyopathy by normalizing ET pathway and expression of FKBP12.6 and SERCA2a in streptozotocin-rats

Min-You Qi, Hao-Ran Liu, De-Zai Dai, Na Li and Yin Dai

Abstract

Total triterpene acids (TTAs) isolated from *Cornus officinalis* Sieb., one of the herbs contained in Liuwei Dihuang decoction, were aimed at alleviating diabetic cardiomyopathy. We hypothesized that the benefits of TTAs may result from suppressing the endothelin–reactive oxidative species (ET-ROS) pathway in the myocardium. Diabetes was produced by a single injection of streptozotocin (STZ, 60 mg kg^{-1}, i.p.) in rats. Assessment of cardiac function, calcium handling proteins, endothelin-1 (ET-1) and redox system was conducted 8 weeks after STZ injection. Medication with TTAs (50 mg kg^{-1}, i.g.) was installed in the last 4 weeks. The compromised cardiac function was characterized by depressed contractility (LVSP and LV+dp/dt_{max}) and relaxation (LVEDP and –LVdp/dt_{min}) in association with hyperglycaemia (30.2 ± 2.6 mmol L^{-1}) in STZ-injected rats. Down-regulated expression of FKBP12.6 (calstabin 2), sarcoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) and phospholamban (PLB) were also found. These changes occurred in connection with an increased ET-1, up-regulated mRNA of propreET-1 and endothelin converting enzyme (ECE), and a state of oxidant stress was found by increased malondialdehyde (MDA), decreased superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) activity, and an enhanced activity and expression of inducible nitric oxide synthase (iNOS) in the diabetic myocardium. After 4 weeks of treatment with TTAs, these changes were alleviated dramatically despite a mild reduction in hyperglycaemia (26.9 ± 3.4 mmol L^{-1}). In conclusion, TTAs, as active ingredients of Liuwei Dihuang decoction, alleviated diabetic cardiomyopathy by normalizing the abnormality of FKBP12.6 and SERCA2a and ET-ROS pathway in the myocardium rather than by hypoglycaemic activity.

Research Division of Pharmacology, China Pharmaceutical University, Nanjing, Jiangsu, 210009, China

Min-You Qi, Hao-Ran Liu, De-Zai Dai, Na Li, Yin Dai

Department of Pharmacology, College of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou, Zhejiang, 310014, China

Min-You Qi

Department of Pharmacy, College of Chemistry and Chemical Engineering, Hu'nan University, Changsha, Hu'nan, 410082, China

Hao-Ran Liu

Correspondence: D.-Z. Dai, Research Division of Pharmacology, China Pharmaceutical University, Nanjing, Jiangsu, 210009, China.
E-mail: dezaidai@vip.sina.com

Acknowledgements and funding: This study was supported by the National Basic Research Program of China (973 Program: 2007CB512000/2007CB512006, to BF Yang). The authors thank Xia Hui-jin and Su Wei for their technical assistance in conducting the project.

Introduction

Diabetic cardiomyopathy is an important complication in patients with diabetes (Sander et al 2004) and manifests as hypertrophied ventricle and compromised cardiac function, likely attributed to defective Ca^{2+} handling proteins in the sarcoplasmic reticulum (Qi et al 2006). In cardiomyocytes, the ryanodine receptor 2 (RyR2), a calcium-releasing channel facing L-type channels at the transverse tube, associates with the main modulator calstabin 2 (FKBP12.6), normal expression of which is essential in maintaining normal function of calcium release from RyR2. Sarcoplasmic/endoplasmic reticulum-Ca^{2+}-ATPase 2a (SERCA2a) pumping calcium back to the sarcoplasmic reticulum is regulated by phospholamban (PLB) dependent on status of phosphorylation. An enhancement of protein kinase C (PKC) (Yaras et al 2007) and protein kinase A (PKA) (Na et al 2007b) in the myocardium contributes to depressed calcium handling ability leading to compromised cardiac function and an increased risk for cardiac arrhythmias. Accumulated evidence suggests that an excessive activation of the endothelin-1 (ET-1) system may associate with reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS), playing important roles in pathological progressions of diabetic myocardium (Singal et al 2001). Hyperglycaemia is a potent stimulus for production of advanced glycation end products (AGEs), which serve as free radicals to damage cardiomyocytes and generate an excess of ET-1. An abnormal ET-1 system directly impairs cardiac contractility and induces proliferation of the myocardium resulting from an abnormal calcium handling system (Qi et al 2006). A rise in intracellular calcium

produces harm to cardiomyocytes and endothelium causing further genesis and release of ET-1 in the presence of sustained hyperglycaemia that in turn promotes ROS generation in diabetes. Oxidative stress predominantly underlies the pathology of diabetic cardiomyopathy, thus both the ET system (Chen et al 2000) and oxidant stress (Singal et al 2001; Wold & Ren 2004) are targets for drug intervention in relieving the cardiac complications of diabetes.

Current therapy to treat diabetes and its complications targets the downstream events subsequent to hyperglycaemia. Traditional Chinese medicines (TCM) used to treat diabetes differ from conventional anti-diabetic agents by improving the general function of the body rather than just focusing on hypoglycaemic activity. There is a famous classic decoction of TCM (a complex decoction referred to as Liuwei Dihuang decoction, which is composed of 6 herbal medicines, including Rehmannia (Dihuang) and Fructus Corni), that has been successfully applied in treating diabetes for a long time. However, only recently has the mode of its action in attenuating cardiomyopathy in diabetic rats been investigated (He et al 2007a). Fructus Corni, one of the 6 herbal medicines contained in Liuwei Dihuang decoction and its ethanol extract, possesses numerous pharmacological properties: lowering serum glucose levels, antioxidant, anti-inflammatory and anti-cancer activity (Liou et al 2004; Lee et al 2006). However, the identity of the active ingredient of the classic Liuwei Dihuang decoction responsible for relieving diabetes is uncertain. We have reported that total triterpene acids (TTAs) isolated from Fructus Corni reverse diabetic retinopathy and vasculopathy resulting from suppression of the ET system and iNOS (Su et al 2007) in streptozotocin (STZ) treated-rats. Thus, we hypothesized that the impairment in haemodynamics of diabetic cardiomyopathy induced in STZ-treated rats might be reversed by the effects of TTAs in normalizing the depressed expression of FKBP12.6, SERCA2a and PLB. TTAs confer protection against the adverse reactions to hyperglycaemia by suppressing the ET-1 pathway and an excess of ROS in STZ-induced diabetic rat myocardium. Thus, TTAs likely normalize the down-regulation of the calcium handling system to improve cardiac function, which may result from suppressing endothelin release and by reducing oxidative stress, rather than hyperglycaemia-lowering actions in STZ-injected rats.

Materials and Methods

Agents

Streptozotocin (STZ) was a product of MP Biomedicals Inc. (Lot. No. 2126F). Total triterpene acids of *Cornus officinalis* Sieb (TTAs), presented as yellow-white power, were prepared by the Research Division of Pharmacology, China Pharmaceutical University.

Animals

Male Sprague–Dawley rats, 250–280 g, were purchased from the Animal Center of Nanjing Medical University. This study was approved by the Institutional Animal Care and Use Committee of Jiangsu province and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (1996).

Preparation of total triterpene acids of *Cornus officinalis* Sieb (TTAs)

The plant materials of Fructus Corni (*Cornus officinalis* Sieb) were purchased from the Nanjing Medicinal Plant Commercial Co. Ltd and were split into small blocks of an appropriate size. Upon being dried at 60°C for 12 h to reach a constant weight, the materials were extracted twice with ether (800 mL per 100 g material) for 1.5 h. The extract was filtered and concentrated under reduced pressure, and dried at a lower temperature under vacuum to finally obtain a yellow-white powder (Su et al 2007). The extract mainly consisted of TTAs, of which the yield rate was 0.5% in dry weight of Fructus Corni.

The product was subjected to testing with Libemann reaction reagents, and the results showed that triterpenes were present in the extract. The extract was analysed by a high-performance liquid chromatography-diode array detection (HPLC-DAD) method. The chromatographic separation was conducted on a Nova-pakC18 (250 mm × 4.6 mm, 4 μm) column in Agilent 1100 chromatography system (Agilent Technologies). The mobile phase was a gradient of CH_3CN–0.4% H_3PO_4–H_2O buffer (Wang et al 2004). The results at different wavelengths were compared and an HPLC chromatogram was established based on the data at 210 nm. In the chromatogram, 10 peaks were marked (Figure 1). Among them, peaks numbered 4, 8 and 9 were identified as loganin, oleanolic acid and ursolic acid, respectively. The other peaks were unidentified.

Streptozotocin-induced diabetic rats

Male Sprague–Dawley rats, 250–280 g, were medicated by a single injection of STZ (60 mg kg^{-1}, i.p. in citrate buffer, pH 4.5). Rats' blood sugar was checked every week by sampling from the post-eyeball vein plexus and those with sustained blood glucose over 16.7 mmol L^{-1} were recognized as diabetic. Each day, rats were fed 15 g chow each in a rigid manner for the purpose of achieving an increase in survival of STZ-treated rats in the period of 8 weeks. At the beginning of the 5th week, rats were randomly divided into three groups:

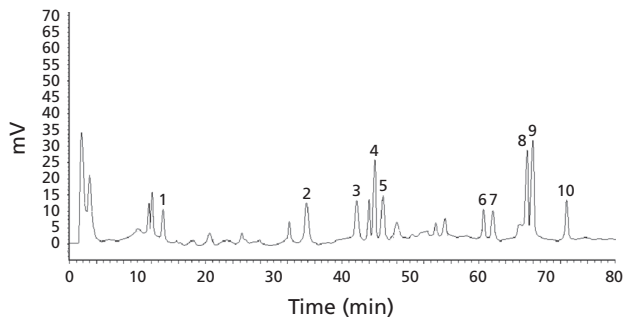

Figure 1 The chromatogram of total tripterpene acids from *Cornus officinalis* Sieb. Peaks numbered 4, 8 and 9 were identified as loganin, oleanolic acid and ursolic acid, respectively.

control, diabetes, diabetes + TTAs (50 mg kg^{-1}, i.g.). A group of STZ-treated rats was continuously medicated with TTAs for 4 weeks and studies of the haemodynamics and measurements of biochemical changes and expression of molecular targets were carried out at the beginning of the 9th week.

Haemodynamics and cardiac weight index

Rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and a polyethylene catheter (PE 50, i.d. 0.58 mm, o.d. 0.965 mm; Becton Dickinson & Co., San Jose, CA, USA) was inserted into the left ventricle via the left common carotid connected to a pressure transducer in a computerized system (MPA-V; the Second Military Medical University, Shanghai, China). The left ventricle (LV) systolic pressure (LVSP), LV+dp/dtmax, the LV end diastolic pressure (LVEDP), LV−dp/dtmin and the heart rate were monitored. After completion of the experiment, the heart was harvested rapidly and rinsed with ice-cold normal saline. The whole heart and the LV, including the septum (SP), were weighed and a ratio to body weight was produced as: the heart weight/body weight (HW/BW, mg/g) and left ventricle weight/body weight (LVW/ BW, mg/g). The left ventricular mass was stored in liquid nitrogen before uses and blood collected was centrifuged at 3000 rev min^{-1} to separate serum for biochemical assay.

Biochemical measurements

The ET-1 and insulin concentration in serum were assessed by radioimmunoassay using the kit (Beijing Northern Bioengineering Institute, China) according to the previous report (Qi et al 2006). The level of blood glucose and myocardial malondialdehyde (MDA) and nitric oxide (NO), and activity of superoxide dismutase (SOD), glutathione peroxidase (GSH- Px), iNOS and cNOS were measured following instructions by the kit manufacturer (Jianchen Biochemical & Bioengineering Co., Nanjing, China) (Tang et al 2005).

RT-PCR of RyR2, FKBP12.6, SERCA2a, PLB, ECE, ppET and iNOS mRNA

The total RNA was isolated from 100 mg frozen ventricular mass by using 1 mL Trizol (BBI, Kitchener, Canada). The amount of total RNA was determined spectrometrically (Gene Ltd, Shanghai, China) at a wavelength of 260 nm and stored at −80°C.

The PCR product of RyR2 and FKBP12.6 (Sakai et al 2000), and SERCA2a, PLB (phospholamban), ECE (endothelin-converting enzyme), ppET (prepro-ET-1) and iNOS mRNA (Na et al 2007b) expression was assessed by a ratio to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR procedure was performed in 25 μL final volume with the following profile: pre-denaturation 5 min at 94°C, denaturation 40 s at 94°C, annealing 40 s at 64°C for SERCA2a, at 55°C for PLB, at 57°C for RyR2, at 53°C for FKBP12.6, at 54°C for ECE, at 55°C for pp ET-1, at 55°C for iNOS and at 63°C for GAPDH, extension 1 min at 72°C. The number of cycles for SERCA2a, PLB, RyR2, FKBP12.6, ECE, ppET, iNOS and GAPDH was 38, 30, 32, 32, 33, 34, 34 and 34, respectively, and a final extension step of 10 min at 72°C followed. Gel electrophoresis was performed and the gel was stained by ethidium bromide individually for semi-quantitative analysis (GDS8000; Syngene, Cambridge, UK).

Western blotting of FKBP12.6, SERCA2a and PLB

SERCA2a, PLB and FKBP12.6 protein levels (Na et al 2007a, b) were performed for quantitative analysis in the myocardium, as briefly described below. An amount of the LV tissue (100 mg) was homogenized in lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg mL^{-1}PMSF, 1 mg mL^{-1}aprotinin, 1% NP-40, 0.5% sodium deoxycholate) using a tissue homogenizer. The lysate was analysed for protein content using a Bradford assay (Bio-Rad, CA, USA). an equal amount of protein was resolved under reducing conditions on 10% SDS-polyacrylamide gel for SERCA2a and 15% SDS-polyacrylamide gel for PLB and FKBP12.6. Immunoblotting was performed using the first antibody to SERCA2a, PLB and FKBP12.6 (Santa Cruz Biotechnology) separately at a dilution of 1:1000 in non-fat milk–Tris buffer. The extract mainly was subsequently probed with a secondary anti-goat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 and detected with 0.1% 3,3′-diaminobenzidine (DAB)–0.01% H_2O_2. The bands were analysed by Labworks imaging acquisition and analysis software.

Statistical analysis

All data are expressed as mean ± s.d. and the one-way analysis of variance assay was performed. Differences between two groups were compared by the Student Newman–Keul's test. $P < 0.05$ was considered as statistically significant.

Results

Blood glucose and insulin

Following STZ injection a significant elevation of blood glucose was found. After 8 weeks it was measured as 363.9% relative to normal ($P < 0.01$) in association with a reduced insulin in serum, down to 36.4% ($P < 0.01$) of normal. Treatment with TTAs in the second 4 weeks produced a moderate increase in serum insulin and a mild decrease in blood glucose ($P < 0.05$) compared with STZ-injected rats (Table 1). However, hyperglycaemia was still very high relative to normal.

Haemodynamics

The systolic and diastolic function were compromised significantly in diabetic rats, with a decrease in LVSP by 23.9% ($P < 0.01$), LV+dp/dt_{max} by 58.6% ($P < 0.01$), an elevated LVEDP by 197.6% ($P < 0.01$), and decreased LV−dp/dtmin by 53.1% ($P < 0.01$), relative to control. An improvement of these changes by TTAs was significant as compared with STZ rats ($P < 0.01$) (Table 1).

Table 1 Cardiac weight index, serum insulin and glucose, and haemodynamics were changed by streptozotocin medication of rats and responded to intervention with TTAs (50 mg kg^{-1}, i.g.)

Parameter	Control	Diabetic	Diabetic + TTAs
Body weight (g)	324.8 ± 27.3	202.5 ± 31.0**	271.9 ± 13.1##
Heart weight/body weight (mg/g)	2.5 ± 0.2	3.3 ± 0.3**	3.0 ± 0.2##
Left ventricle weight/body weight (mg/g)	1.7 ± 0.1	2.5 ± 0.6**	2.0 ± 0.1#
Blood insulin (mIU L^{-1})	61.2 ± 16.9	22.3 ± 9.2**	37.1 ± 10.1#
Blood glucose (mmol L^{-1})	8.3 ± 1.2	30.2 ± 2.6**	26.9 ± 3.4#
LVSP (mmHg)	128.2 ± 19.3	97.6 ± 13.5**	113.5 ± 12.1#
LVEDP (mmHg)	9.8 ± 2.5	29.2 ± 4.9**	10.3 ± 0.6##
LV+dp/dtmax (mmHg/s)	6454.2 ± 578.0	2673.9 ± 583.9**	5837.6 ± 537.0##
LV−dp/dtmin (mmHg/s)	4514.9 ± 677.5	2115.4 ± 263.9**	3907.5 ± 142.4##

Data are presented as means ± s.d., n = 8. $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetic rats.

Cardiac hypertrophy

Cardiac hypertrophy was assessed by measuring the cardiac weight against the body weight in rats. The LV weight index and heart weight index were increased by 50.8% ($P < 0.01$) and 32.0% ($P < 0.01$) in diabetic cardiomyopathy relative to normal, respectively. This indicated a significant ventricular hypertrophy caused by sustained hyperglycaemia following STZ injection. Treatment with TTAs, along with a relief in cardiac performance, reduced ventricular remodelling significantly ($P < 0.01$) (Table 1).

Abnormal expression of SERCA2a, PLB and FKBP12.6

Normal expression of calcium handling proteins is critical in maintaining normal cardiac pumping activity. There was significant depression in mRNA expression of the Ca^{2+} handling system in the diabetic heart. The abundance of mRNA of SERCA2a, PLB, RyR2, and FKBP12.6 was respectively decreased by 63.9%, 26.9%, 54.2% and 20.8% ($P < 0.01$) in the LV of diabetic rats compared with the normal rats. TTAs were effective in recovering the down-regulated abundance of mRNA completely ($P < 0.01$) (Figure 2A).

Proteins of SERCA2a, PLB and FKBP12.6 were targeted further to support the changes in mRNA. Significant decrease in proteins of SERCA2a, PLB and FKBP12.6 was found, by 45.2%, 35.6% and 35.1% in the LV ($P < 0.01$) relative to normal, respectively. Thus, it was confirmed that the cardiac insufficiency of diabetic rats was attributed to down-regulation of calcium modulating proteins, which adversely affects both the activity of Ca^{2+} release and uptake by the sarcoplasmic reticulum. Treatment with TTAs significantly increased the down-regulated protein levels as compared with the STZ-treated rat group (Figure 2B).

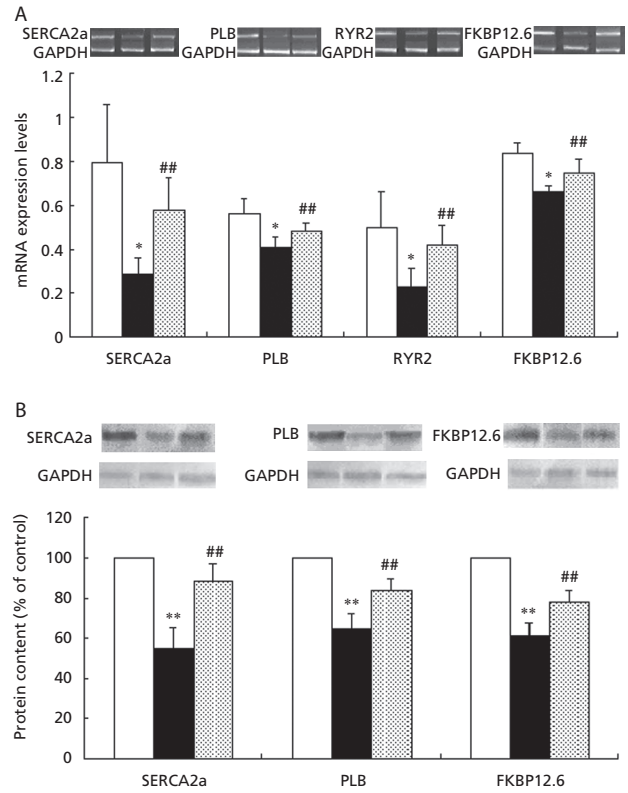

Figure 2 Down-regulation of mRNA and protein expressions of the Ca^{2+} handing system of the sarcoplasmic reticulum was found in the myocardium in STZ-injected rats and was normalized by intervention with TTAs (50 mg kg^{-1}, i.g.) significantly. A. mRNA. Control (open columns), diabetic (black columns) and TTA-treated diabetic heart (grey columns). The upper panel shows mRNA bands from a typical record. B. Protein levels. The upper panel shows protein bands from a typical record and GAPDH as loading controls. (Molecular weight of different proteins: SERCA2a 110 kDa; PLB 22 kDa; FKBP12.6 12 kDa). Means ± s.d., n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus diabetic.

Activated endothelin pathway

Serum ET-1 concentrations were elevated significantly in diabetic rats by 71.3% compared with the non-diabetic controls ($P < 0.01$). mRNA abundance of ECE and ppET-1 in the diabetic LV was remarkably up-regulated relative to the control ($P < 0.01$). Treatment with TTAs significantly reduced ET-1 levels ($P < 0.01$) and abundance of mRNA toward normal, respectively (Figure 3A, B).

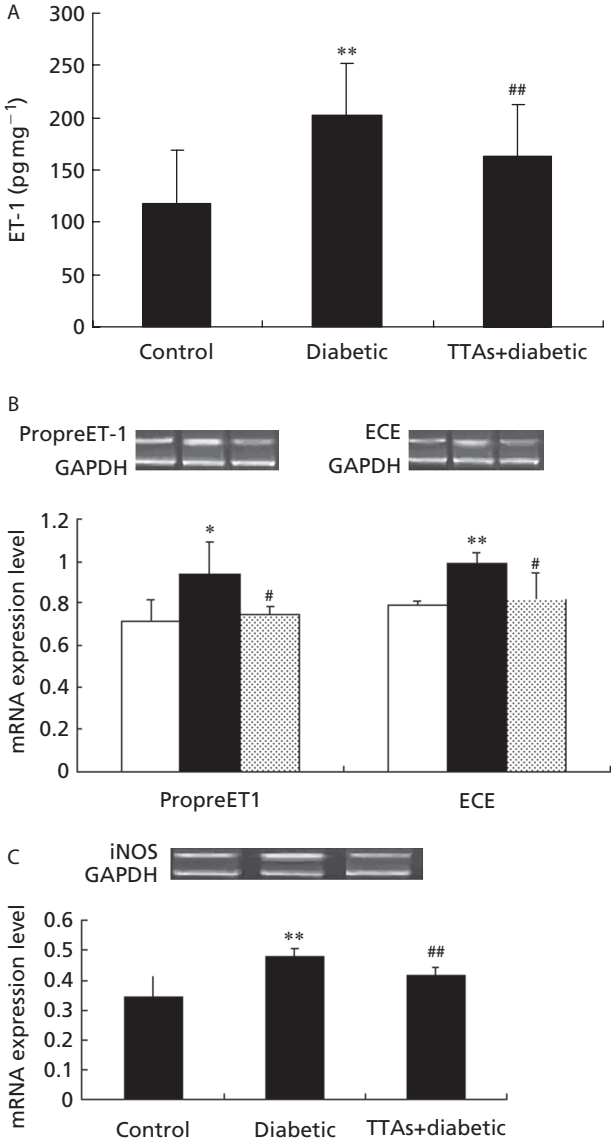

Figure 3 Increased ET-1 content and mRNA expression of ECE, preproET-1 and iNOS were found in the myocardium in STZ-injected rats and reversed by TTAs (50 mg kg^{-1}, i.g.). A. Plasma ET-1. B. ECE and ppET-1 in the control (open columns), diabetic (black columns) and TTA-treated diabetic heart (grey columns), respectively. The upper panel shows bands from a typical experiment. C. iNOS mRNA. Means ± s.d., n = 5–8. *$P < 0.05$, **$P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetic.

Exaggerated oxidative stress

MDA content was increased by 28% ($P < 0.01$), SOD decreased by 25.0% ($P < 0.01$) and GSH-Px decreased by 33.7% ($P < 0.01$) in diabetic heart relative to control. Abnormalities of MDA, SOD and GSH-Px in the myocardium were abolished following the intervention with TTAs ($P < 0.01$, $P < 0.05$) (Table 2).

Abnormal NOS activity and NO content

An increase in ET-1 impairs the biosynthesis and release of NO in the endothelium in diabetic rats. Indeed, NO content was reduced sharply by 41.3% ($P < 0.01$) and the activity of the tNOS (total NOS) and cNOS was also depressed by 23.2% and 39.6% ($P < 0.01$) relative to the control, respectively (Table 3).

Table 2 MDA production, SOD and GSH-Px activity in the myocardium of rats were changed by streptozotocin medication and responded to intervention with TTAs (50 mg kg^{-1}, i.g.)

Group	MDA (nmol (mg protein)$^{-1}$)	SOD (U (mg protein)$^{-1}$)	GSH-Px (U (mg protein)$^{-1}$)
Control	6.90 ± 0.84	129.69 ± 10.49	28.97 ± 4.01
Diabetes	8.84 ± 0.75**	97.24 ± 9.66**	19.19 ± 3.37**
TTAs + diabetes	7.52 ± 0.74$^{\#\#}$	114.2 ± 5.56$^{\#}$	24.77 ± 5.62$^{\#}$

Data are presented as mean ± s.d., **$P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetes.

The induced NOS activity is considered to be an important factor underlying the status of oxidative stress; the iNOS activity was increased by 50.5% ($P < 0.01$) (Table 3) in association with an up-regulation of iNOS mRNA ($P < 0.01$) in diabetic cardiomyopathy relative to normal and treatment with TTAs significantly reduced the STZ-induced changes in NO, iNOS activity and mRNA ($P < 0.05$) (Figure 3C).

Discussion

Diabetic cardiomyopathy is characterized by marked ventricular remodelling in conjunction with reduced cardiac performance, which are due to diabetic-induced insults to the myocardium independent of vascular complications, but attributed to defective calcium handling protein SERCA2a (Vetter et al 2002). An increase in cardiac weight index reflects proliferation of cardiomyocytes, which results from an activation of the ET-1 pathway (Drimal et al 1999; Turner et al 2004). Dysfunctional contractility and relaxation of diabetic cardiomyopathy are predominant, resulting from detrimental effects of hyperglycaemia on mechanics. Adverse effects by sustained hyperglycaemia on the contractility and relaxation of the affected heart were reversed dramatically in TTA-treated rats. The efficacy of TTAs to recover cardiac performance towards normal is comparable with that of the endothelin receptor antagonist bosentan (Verma et al 2002) and the dual endothelin receptor antagonist CPU0213 (Qi et al 2006). The effect is also in line with those of Liuwei Dihuang decoction (He et al 2007a) in which Fructus Corni is only one of six crude herbal medicines. Interestingly, we proved in this study that the effects of TTAs on diabetic cardiomyopathy are equivalent to those of the whole six-element decoction (He et al 2007a). The beneficial effects of TTAs on cardiac insufficiency are not based on a glucose-lowering action as this was only mild and not sufficient to alleviate hyperglycaemia. In contrast, an effect in normalizing cardiac function and expression of calcium handling was almost complete. Thus, we suggest that the effects of TTA on diabetic cardiac disease result from a

Table 3 NO content and NOS activity in the myocardium of rats were changed by streptozotocin medication and responded to intervention with TTAs (50 mg kg^{-1}, i.g.)

Group	NO (μmol (g protein)$^{-1}$)	tNOS (U (mg protein)$^{-1}$)	iNOS (U (mg protein)$^{-1}$)	cNOS (U (mg protein)$^{-1}$)
Control	1.90 ± 0.59	0.77 ± 0.11	0.14 ± 0.03	0.63 ± 0.09
Diabetes	1.11 ± 0.35**	0.59 ± 0.09**	0.21 ± 0.03**	0.38 ± 0.07**
TTA + diabetes	1.47 ± 0.25$^{\#\#}$	0.66 ± 0.05$^{\#}$	0.17 ± 0.01$^{\#\#}$	0.49 ± 0.05$^{\#\#}$

Data are presented as mean ± s.d., n = 8. $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus diabetes.

blockade of events downstream of the pathway activated by sustained hyperglycaemia, rather than a hypoglycaemic effect.

Cardiac dysfunction caused by lesions in the myocardium is attributable to an abnormal Ca^{2+} handling system, which changes the sarcoplasmic reticulum calcium release and uptake resulting in elevated cystolic calcium levels during diastole (Yano et al 2003; Wehrens & Marks 2004). The compromised cardiac function at systole is likely due to diminished Ca^{2+} released from the sarcoplasmic reticulum where less Ca^{2+} is stored with calsequestrin and is associated with a calcium leak at diastole leading to failing heart (Chopra et al 2007; Shannon 2007). Dysfunction of relaxation correlates to an elevated cytoplasmic calcium at diastole, which can be affected by at least two main factors (i.e. sharply stopping Ca^{2+} release from RyR2, which is critically affected by down-regulation of FKBP12.6 (Na et al 2007b), and Ca^{2+} pumping activity of SERCA2a (Cheng et al 2007; Na et al 2007a), of which down-regulation of its mRNA and proteins at sarcoplasmic reticulum is linked to cardiac failure). Increased phosphorylation of RyR2 may provide a situation predisposing to dissociation of FKBP12.6, resulting in unstable RyR2 channels, which manifests as calcium leak to retard declining calcium and remaining elevated calcium levels in diastole (Marks 2003). Changes in diastolic calcium were shown in cardiomyocytes isolated from failing heart (Shannon et al 2003) and underlie distorted electrophysiological processes in the myocardium, manifesting as repolarization abnormality that predisposes the affected heart to risk of sudden cardiac death (Lehnart et al 2004).

Diabetes and heart failure commonly occur together and result in a serious medical problem with poor outcome (Zhang et al 2006; Erdmann & Wilcox 2008). The factors contributing to heart failure are multi-factorial; however, diabetic cardiomyopathy and arrhythmias are major concerns and an increase in risk of severe arrhythmias is a matter of concern. Patients with diabetes show long QT syndrome in ECG recordings, which has become a non-negligible clinical problem and has attracted an increasing attention for basic investigation because it increases the risk of lethal ventricular arrhythmias (Ding et al 2006). These phenomena can be reversed by effective therapy, including insulin and other agents. The development of ion channelopathy is likely due to insults from hyperglycaemia to the sarcolemma by affecting the pathway that may impact on ion channels at both the sarcolemma (such as IKr channel encoded by KCNH2 and L-type Ca^{2+} channel encoded by Cav1.2 gene) and sarcoplasmic reticulum (RyR2, FKBP12.6, SERCA2a and PLB). An abnormal ET-1 pathway, which develops in the presence of hyperglycaemia, is critically involved in the arrhythmogenic property of diabetic heart by down-regulation of IKr, IKs and Ito, which can by relieved by intervention with the endothelin receptor antagonist bosentan (Ding et al 2006). Down-regulation of FKBP12.6, SERCA2a and PLB is likely engaged in the arrhythmogenetic property in diabetic myocardium. Dissociation/down-regulation of FKBP12.6 is the determinant to induce calcium leak from an unstable RyR2, which can be caused by β-receptor stimulation (Wehrens et al 2003). It is true, as we have proved, that in-vivo treatment with isoproterenol results in defective cardiac contractility and relaxation associated with the protein FKBP12.6 dissociation from its binding site on the ryanodine receptor into the cytosol and re-association following treatment with the endothelin receptor antagonist CPU0213 (Feng et al 2007). Thus, dissociation of FKBP12.6 is an important target for new drug discovery in relieving both cardiac insufficiency (Yano et al 2003; Feng et al 2007; He et al 2007b) and cardiac arrhythmias (Doggrell 2005; Lehnart et al 2006). In this study we have shown that TTAs were effective in reversing the down-regulation of FKBP12.6, SERCA2a and PLB and normalizing the abnormal cardiac function of STZ-induced diabetic rats and, potentially, may relieve the cardiac failure and arrhythmogenesis associated with the diabetic heart.

Normal expression of SERCA2a is critical in maintaining normal cardiac contractility. SERCA2a activity and expression are well controlled by PLB and both SERCA2a and PLB are down-regulated in diabetic cardiomyopathy, data that is comparable with previous reports (Penckofer et al 2002; Qi et al 2006). SERCA2a and PLB protein levels were concomitantly decreased in the failing heart, but, despite sustained hyperglycaemia, SERCA2a and PLB levels were restored to the normal range following treatment with TTAs.

Oxidative damage is widely re-considered to be an aetiological factor in cardiovascular diseases in general and involved in diabetic complications in particular (Likidlilid et al 2007). Over-production of ROS initiates long-term development of diabetic complications (Kiritoshi et al 2003). Excessive generation of highly reactive free radicals, largely secondary to hyperglycaemia, causes oxidative stress to further exacerbate progression of diabetic cardiomyopathy. A crosstalk between ROS and ET-1 has been well established in many studies (Xu et al 2004). In this study we demonstrated elevated oxidative stress, as reflected by an increased production of MDA and a diminished activity of antioxidant SOD and GSH-Px accompanied by augmented ET-1 levels and up-regulated mRNA for ppET-1 and ECE, in the diabetic heart. An antioxidant effect of TTAs is consistent with the findings with ethanol extracts of Fructus Corni (Lee et al 2006). A specific NFκB promoter module is activated in the inflammatory stress response contributing to an up-regulation of a paracrine

ET-1 in progressive diabetes (Schmid et al 2006). Indeed, an up-regulation of the ET system correlates to an increase in ROS, which diffuse into the nucleus to activate NFκB and to promote the transcription process in a vicious cycle leading to up-regulation of mRNA for prepro-ET-1 and iNOS (Xia et al 2006). A genesis of iNOS reinforces oxidant stress actively involved in the pathogenesis of diabetes (Kaneki et al 2007). Thus, based on these findings, S-nitrosylation has recently been proposed to play an important role in insulin resistance. A significant suppression of iNOS may indicate the potential effectiveness of TTAs to relieve insulin resistance in type II diabetes. The cNOS activity and NO content are depressed, likely reflecting a reduction in vascular relaxation, which can be reversed by TTAs as in line with previous findings (Su et al 2007). Thus, an anti-oxidation action of TTAs is likely a key action that results in the normalization of the ET-1 pathway, correcting intracellular calcium handling and cardiac mechanics in diabetic cardiomyopathy.

Conclusion

The objective of this study was to determine the activity of total triterpene acids, TTAs, isolated from Corni Fructus as active ingredients of Liuwei Dihuang decoction in treating cardiac cardiomyopathy. The results obtained with TTAs were comparable with those previously reported for the whole six-element complex decoction and those of endothelin antagonists. Thus, by suppressing the ET pathway and ROS production, TTAs were effective in alleviating diabetes-induced cardiomyopathy, and these beneficial effects can also be linked to normalizing the expression of the sarcoplasmic reticulum calcium release and uptake proteins FKBP12.6, SERCA2a and phospholamban, but were independent of any action to reverse hyperglycaemia.

References

Chen, S., Evans, T., Mukherjee, K., Karmazyn, M., Chakrabarti, S. (2000) Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. *J. Mol. Cell. Cardiol.* **32**: 1621–1629

Cheng, Y., George, I., Yi, G. H., Reiken, S., Gu, A., Tao, Y. K., Muraskin, J., Qin, S., He, K. L., Hay, I., Yu, K., Oz, M. C., Burkhoff, D., Holmes, J., Wang, J. (2007) Bradycardic therapy improves left ventricular function and remodeling in dogs with coronary embolization-induced chronic heart failure. *J. Pharmacol. Exp. Ther.* **321**: 469–476

Chopra, N., Kannankeril, P. J., Yang, T., Hlaing, T., Holinstat, I., Ettensohn, K., Pfeifer, K., Akin, B., Jones, L. R., Franzini-Armstrong, C., Knollmann, B. C. (2007) Modest reductions of cardiac calsequestrin increase sarcoplasmic reticulum Ca2+ leak independent of luminal Ca2+ and trigger ventricular arrhythmias in mice. *Circ. Res.* **101**: 617–626

Ding, Y., Zou, R., Judd, R. L., Zhong, J. (2006) Endothelin-1 receptor blockade prevented the electrophysiological dysfunction in cardiac myocytes of streptozotocin-induced diabetic rats. *Endocrine* **30**: 121–127

Doggrell, S. A. (2005) Stabilisation of calstabin2—a new approach in sudden cardiac death. *Expert. Opin. Ther. Targets* **9**: 955–962

Drimal, J., Mislovicova, M., Ismail, A., Moncek, F. (1999) Detrimental subtype-specific endothelin-1 (ET-1) signaling in myocardial cells: the ET(A) mediated proliferation and ET(B) receptor down-regulation. *Physiol. Res.* **48**: 9–19

Erdmann, E., Wilcox, R. G. (2008) Weighing up the cardiovascular benefits of thiazolidinedione therapy: the impact of increased risk of heart failure. *Eur. Heart J.* **29**: 12–20

Feng, Y., Tang, X. Y., Dai, D. Z., Dai, Y. (2007) Reversal of isoproterenol-induced downregulation of phospholamban and FKBP12.6 by CPU0213-mediated antagonism of endothelin receptors. *Acta Pharmacol. Sin.* **28**: 1746–1754

He, H., Yang, X., Zeng, X., Shi, M., Yang, J., Wu, L., Li, L. (2007a) Protective effect of Liuwei Dihuang decoction on early diabetic nephropathy induced by streptozotocin via modulating ET-ROS axis and matrix metalloproteinase activity in rats. *J. Pharm. Pharmacol.* **59**: 1297–1305

He, H. B., Yu, F., Dai, D. Z., Dai, Y. (2007b) Down-regulation of FKBP12.6 and SERCA2a contributes to acute heart failure in septic shock and is related to an up-regulated endothelin signalling pathway. *J. Pharm. Pharmacol.* **59**: 977–984

Kaneki, M., Shimizu, N., Yamada, D., Chang, K. (2007) Nitrosative stress and pathogenesis of insulin resistance. *Antioxid. Redox Signal.* **9**: 319–329

Kiritoshi, S., Nishikawa, T., Sonoda, K., Kukidome, D., Senokuchi, T., Matsuo, T., Matsumura, T., Tokunaga, H., Brownlee, M., Araki, E. (2003) Reactive oxygen species from mitochondria induce cyclooxygenase-2 gene expression in human mesangial cells: potential role in diabetic nephropathy. *Diabetes* **52**: 2570–2577

Lee, S. O., Kim, S. Y., Han, S. M., Kim, H. M., Ham, S. S., Kang, I. J. (2006) Corni fructus scavenges hydroxy radicals and decreases oxidative stress in endothelial cells. *J. Med. Food* **9**: 594–598

Lehnart, S. E., Wehrens, X. H., Laitinen, P. J., Reiken, S. R., Deng, S. X., Cheng, Z., Landry, D. W., Kontula, K., Swan, H., Marks, A. R. (2004) Sudden death in familial polymorphic ventricular tachycardia associated with calcium release channel (ryanodine receptor) leak. *Circulation* **109**: 3208–3214

Lehnart, S. E., Terrenoire, C., Reiken, S., Wehrens, X. H., Song, L. S., Tillman, E. J., Mancarella, S., Coromilas, J., Lederer, W. J., Kass, R. S., Marks, A. R. (2006) Stabilization of cardiac ryanodine receptor prevents intracellular calcium leak and arrhythmias. *Proc. Natl Acad. Sci. USA* **103**: 7906–7910

Likidlilid, A., Patchanans, N., Poldee, S., Peerapatdit, T. (2007) Glutathione and glutathione peroxidase in type 1 diabetic patients. *J. Med. Assoc. Thai.* **90**: 1759–1767

Liou, S. S., Liu, I. M., Hsu, S. F., Cheng, J. T. (2004) Corni fructus as the major herb of Die-Huang-Wan for lowering plasma glucose in Wistar rats. *J. Pharm. Pharmacol.* **56**: 1443–1447

Marks, A. R. (2003) A guide for the perplexed: towards an understanding of the molecular basis of heart failure. *Circulation* **107**: 1456–1459

Na, T., Dai, D. Z., Tang, X. Y., Dai, Y. (2007a) Upregulation of leptin pathway correlates with abnormal expression of SERCA2a, phospholamban and the endothelin pathway in heart failure and reversal by CPU86017. *Naunyn Schmiedeberg's Arch. Pharmacol.* **375**: 39–49

Na, T., Huang, Z. J., Dai, D. Z., Zhang, Y., Dai, Y. (2007b) Abrupt changes in FKBP12.6 and SERCA2a expression contribute to sudden occurrence of ventricular fibrillation on reperfusion and are prevented by CPU86017. *Acta Pharmacol. Sin.* **28**: 773–782

Penckofer, S., Schwertz, D., Florczak, K. (2002) Oxidative stress and cardiovascular disease in type 2 diabetes: the role of antioxidants and pro-oxidants. *J. Cardiovasc. Nurs.* **16**: 68–85

Qi, M. Y., Xia, H. J., Dai, D. Z., Dai, Y. (2006) A novel endothelin receptor antagonist CPU0213 improves diabetic cardiac insufficiency attributed to up-regulation of the expression of FKBP12.6, SERCA2a, and PLB in rats. *J. Cardiovasc. Pharmacol.* **47**: 729–735

Sakai, S., Miyauchi, T., Yamaguchi, I. (2000) Long-term endothelin receptor antagonist administration improves alterations in expression of various cardiac genes in failing myocardium of rats with heart failure. *Circulation* **101**: 2849–2853

Sander, G. E., Wilklow, F. E., Giles, T. D. (2004) Heart failure in diabetes mellitus: causal and treatment considerations. *Minerva Cardioangiol.* **52**: 491–503

Schmid, H., Boucherot, A., Yasuda, Y., Henger, A., Brunner, B., Eichinger, F., Nitsche, A., Kiss, E., Bleich, M., Grone, H. J., Nelson, P. J., Schlondorff, D., Cohen, C. D., Kretzler, M. (2006) Modular activation of nuclear factor-kappaB transcriptional programs in human diabetic nephropathy. *Diabetes* **55**: 2993–3003

Shannon, T. R. (2007) Linking calsequestrin to lumenal control of SR Ca2+ release. *Circ. Res.* **101**: 539–541

Shannon, T. R., Pogwizd, S. M., Bers, D. M. (2003) Elevated sarcoplasmic reticulum Ca2+ leak in intact ventricular myocytes from rabbits in heart failure. *Circ. Res.* **93**: 592–594

Singal, P. K., Bello-Klein, A., Farahmand, F., Sandhawalia, V. (2001) Oxidative stress and functional deficit in diabetic cardiomyopathy. *Adv. Exp. Med. Biol.* **498**: 213–220

Su, W., Dai, D. Z., Liu, H. R., Na, T., Dai, Y. (2007) Upregulated endothelin system in diabetic vascular dysfunction and early retinopathy is reversed by CPU0213 and total triterpene acids from Fructus Corni. *Clin. Exp. Pharmacol. Physiol.* **34**: 1228–1233

Tang, X. Y., Qi, M. Y., Dai, D. Z., Zhang, C. (2005) Effects of CPU 86017 (chlorobenzyltetrahydroberberine chloride) and its enantiomers on thyrotoxicosis induced overactive endothelin-1 system and oxidative stress in rat testes. *Urology* **48**: 455–461

Turner, N. A., O'Regan, D. J., Ball, S. G., Porter, K. E. (2004) Endothelin-1 is an essential co-factor for beta2-adrenergic receptor-induced proliferation of human cardiac fibroblasts. *FEBS. Lett.* **576**: 156–160

Verma, S., Maitland, A., Weisel, R. D., Li, S. H., Fedak, P. W., Pomroy, N. C., Mickle, D. A., Li, R. K., Ko, L., Rao, V. (2002) Hyperglycemia exaggerates ischemia-reperfusion-induced cardiomyocyte injury: reversal with endothelin antagonism. *J. Thorac. Cardiovasc. Surg.* **123**: 1120–1124

Vetter, R., Rehfeld, U., Reissfelder, C., Weiss, W., Wagner, K. D., Gunther, J., Hammes, A., Tschope, C., Dillmann, W., Paul, M. (2002) Transgenic overexpression of the sarcoplasmic reticulum Ca2+ATPase improves reticular Ca2+ handling in normal and diabetic rat hearts. *Faseb. J.* **16**: 1657–1659

Wang, H. H., Guo, W. B., Gu, Y., Wang, Z. Z. (2004) Studies on fingerprints of *Cornus officinalis Sieb. Xi Bei Zhi Wu Xue Bao* **24**: 1922–1928

Wehrens, X. H., Marks, A. R. (2004) Molecular determinants of altered contractility in heart failure. *Ann. Med.* **36** (Suppl. 1): 70–80

Wehrens, X. H., Lehnart, S. E., Huang, F., Vest, J. A., Reiken, S. R., Mohler, P. J., Sun, J., Guatimosim, S., Song, L. S., Rosemblit, N., D'Armiento, J. M., Napolitano, C., Memmi, M., Priori, S. G., Lederer, W. J., Marks, A. R. (2003) FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell* **113**: 829–840

Wold, L. E., Ren, J. (2004) Streptozotocin directly impairs cardiac contractile function in isolated ventricular myocytes via a p38 map kinase-dependent oxidative stress mechanism. *Biochem. Biophys. Res. Commun.* **318**: 1066–1071

Xia, H. J., Dai, D. Z., Dai, Y. (2006) Up-regulated inflammatory factors endothelin, NFkappaB, TNFalpha and iNOS involved in exaggerated cardiac arrhythmias in l-thyroxine-induced cardiomyopathy are suppressed by darusentan in rats. *Life Sci.* **79**: 1812–1819

Xu, F. P., Chen, M. S., Wang, Y. Z., Yi, Q., Lin, S. B., Chen, A. F., Luo, J. D. (2004) Leptin induces hypertrophy via endothelin-1-reactive oxygen species pathway in cultured neonatal rat cardiomyocytes. *Circulation* **110**: 1269–1275

Yano, M., Kobayashi, S., Kohno, M., Doi, M., Tokuhisa, T., Okuda, S., Suetsugu, M., Hisaoka, T., Obayashi, M., Ohkusa, T., Kohno, M., Matsuzaki, M. (2003) FKBP12.6-mediated stabilization of calcium-release channel (ryanodine receptor) as a novel therapeutic strategy against heart failure. *Circulation* **107**: 477–484

Yaras, N., Bilginoglu, A., Vassort, G., Turan, B. (2007) Restoration of diabetes-induced abnormal local Ca2+ release in cardiomyocytes by angiotensin II receptor blockade. *Am. J. Physiol. Heart Circ. Physiol.* **292**: H912–H920

Zhang, Y., Xiao, J., Wang, H., Luo, X., Wang, J., Villeneuve, L. R., Zhang, H., Bai, Y., Yang, B., Wang, Z. (2006) Restoring depressed HERG K+ channel function as a mechanism for insulin treatment of abnormal QT prolongation and associated arrhythmias in diabetic rabbits. *Am. J. Physiol. Heart Circ. Physiol.* **291**: H1446–H1455