Protocatechuic acid exerts a cardioprotective effect in type 1 diabetic rats

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Abstract

Oxidative stress has been shown to play an important role in the pathogenesis of diabetes-induced cardiac dysfunction. Protocatechuic acid (PCA) is a phenolic compound, a main metabolite of anthocyanin, which has been reported to display various pharmacological properties. We proposed the hypothesis that PCA exerts cardioprotection in type 1 diabetic (T1DM) rats. T1DM was induced in male Sprague–Dawley rats by a single i.p. injection of 50 mg/kg streptozotocin (STZ) and groups of these animals received the following treatments for 12 weeks: i) oral administration of vehicle, ii) oral administration of PCA at a dose of 50 mg/kg per day, iii) oral administration of PCA at a dose of 100 mg/kg per day, iv) s.c. injection of insulin at a dose of 4 U/kg per day, and v) a combination of PCA, 100 mg/kg per day and insulin, 4 U/kg per day. Metabolic parameters, results from echocardiography, and heart rate variability were monitored every 4 weeks, and the HbA1c, cardiac malondialdehyde (MDA), cardiac mitochondrial function, and cardiac BAX/BCL2 expression were evaluated at the end of treatment. PCA, insulin, and combined drug treatments significantly improved metabolic parameters and cardiac function as shown by increased percentage fractional shortening and percentage left ventricular ejection fraction and decreased low-frequency:high-frequency ratio in T1DM rats. Moreover, all treatments significantly decreased plasma HbA1c and cardiac MDA levels, improved cardiac mitochondrial function, and increased BCL2 expression. Our results demonstrated for the first time, to our knowledge, the efficacy of PCA in improving cardiac function and cardiac autonomic balance, preventing cardiac mitochondrial dysfunction, and increasing anti-apoptotic protein in STZ-induced T1DM rats. Thus, PCA possesses a potential cardioprotective effect and could restore cardiac function when combined with insulin treatment. These findings indicated that supplementation with PCA might be helpful for the prevention and alleviation of cardiovascular complications in T1DM.

Key Words
- type 1 diabetes
- oxidative stress
- cardiac mitochondrial dysfunction
- protocatechuic acid

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Introduction

Diabetes and cardiac complications have become a public health problem of considerable magnitude. Cardiac dysfunction is the most common serious complication of diabetes mellitus and has become one of the leading causes of the increased mortality in both Type 1 and Type 2 diabetes. A streptozotocin (STZ)-induced type 1 diabetic rat has been used as an animal model for the investigation of pharmacological interventions and also for the study of diabetic complications including diabetes-induced cardiac dysfunction (Krishna *et al.* 2005, Naowaboot *et al.* 2009). Clinically, cardiac dysfunction may occur without major vascular lesions, indicating a primary role for the direct effects of diabetes on cardiomyocytes (Trost & LeWinter 2001, Dyntar *et al.* 2006). These characteristics further result in diabetes-induced cardiac dysfunction through cellular pathological changes, especially increased oxidative stress, mitochondrial dysfunction, and cardiac autonomic neuropathy (An & Rodrigues 2006, Boudina & Abel 2007). Oxidative damage plays an important role in the occurrence and development of cardiac dysfunction in this case (Asbun & Villarreal 2006, Kumar & Sitasawad 2009). Oxidative stress is known to potentially lead to biological damage to macromolecules and activates maladaptive signaling pathways, which may cause cell dysfunction and cell death (Sies 1991).

Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid), a phenolic compound, is isolated from the dried flowers of *Hibiscus sabdariffa* Linn. It is a main metabolite of complex polyphenols, especially anthocyanins, which are converted to PCA and also abundantly formed and absorbed in the large intestine because of microbial metabolization (Kay *et al.* 2005, Vitaglione *et al.* 2007). Previous reports of both *in vitro* and *in vivo* studies have demonstrated that PCA has anti-carcinogen (Tseng *et al.* 2000, Yip *et al.* 2006, Lin *et al.* 2007, Yin *et al.* 2009), anti-hyperglycemia (Lin *et al.* 2009, 2011, Harini & Pugalendi 2010, Scazzocchio *et al.* 2011), antioxidant (Liu *et al.* 2002, Sroka & Cisowski 2003, Masella *et al.* 2004, Shi *et al.* 2006, Tarozzi *et al.* 2007, Lin *et al.* 2009, Chou *et al.* 2010, Vari *et al.* 2011, Zhang *et al.* 2011), and anti-inflammatory properties (Yan *et al.* 2004, Min *et al.* 2010, Wang *et al.* 2010, 2011). However, no scientific investigation has yet been conducted on the effect of PCA on diabetes-induced cardiac dysfunction, which is also caused by oxidative stress, and thus this investigation was conducted to study the anti-hyperglycemic activity of PCA and the effect of PCA on diabetes-induced cardiac dysfunction and other related metabolic parameters in STZ-induced diabetic rats.

We tested the hypothesis that PCA could attenuate cardiac complications by reducing oxidative stress, ameliorating cardiomyocyte apoptosis, and improving the cardiac autonomic balance and cardiac mitochondrial function in STZ-induced diabetic rats.

Materials and methods

Animals

A total of 36 male Sprague–Dawley rats with body weights of 250–280 g were acquired from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. All experiments complied with the standards for the care and use of experimental animals and were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee. After 7 days of acclimatization, diabetes was induced in 30 of the rats by a single i.p. injection of 50 mg/kg body weight of STZ dissolved in 0.1 M citric buffer (pH 4.5). Normal control rats ($n=6$) received injections of citric buffer (vehicle). Ten days after vehicle/STZ injection, only diabetic rats with fasting blood glucose (FBG) over 300 mg/dl were included in the experiments (Jiang *et al.* 2011, Zhao *et al.* 2014). Rats were assigned to one of six groups with six rats in each group and were treated as follows: normal rats treated with vehicle (NMV), diabetic rats treated with vehicle (DMV), diabetic rats treated with insulin at a dose of 4 U/kg (DMI), diabetic rats treated with PCA at a dose of 50 mg/kg (DML), diabetic rats treated with PCA at a dose of 100 mg/kg (DMH) (Harini & Pugalendi 2010), and diabetic rats treated with DMH and insulin at a dose of 4 U/kg (DMHI). All treatments were administered daily for 12 weeks. Fifty percent propylene glycol (vehicle) and PCA (Sigma Chemical Co. Ltd) were administrated orally using gavage feeding, and insulin was injected subcutaneously. The animals were given free access to feed and drinking water. The body weight and food intake were recorded weekly. Echocardiography was performed and heart rate variability (HRV) was measured every 4 weeks and blood sampling from tail vein was performed after fasting for 12 h to measure plasma glucose, insulin, and malondialdehyde (MDA) levels. At the end of a 12-week treatment, animals were deeply anesthetized and then killed; the hearts were collected for investigation of mitochondrial function, MDA, and BAX and BCL2 protein levels, and blood was collected for investigation of HbA1c.

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FBG, plasma insulin, and HbA1c level determination

FBG level was determined using a glucometer (Accu-Chek Advantage II; Roche Diagnostics). Plasma insulin levels were measured by Sandwich ELISA (LINCO Research, Saint Charles, MO, USA; Pratchayasakul *et al.* 2011). Plasma HbA1c was determined based on the competitive turbidimetric inhibition immunoassay (Roche Diagnostics Ltd).

Plasma and cardiac MDA level determination

Plasma and cardiac MDA level was determined using a HPLC-based assay (Thermo scientific, Bangkok, Thailand). Cardiac tissue was homogenized in phosphate buffer, pH 2.8. Plasma and cardiac tissue were mixed with H_3PO_4 and thiobarbituric acid (TBA) to produce TBA-reactive substances (TBARSs). The plasma and cardiac TBARS concentrations were determined from a standard curve and reported as being equivalent to the MDA concentration (Apaijai *et al.* 2013).

Echocardiography

The echocardiographic parameters were measured in each rat using an HP/Agilent Philips Sonos 4500 (Agilent Technologies, Santa Clara, CA, USA). An echocardiography probe was placed in gentle contact with the chest, and images were collected along the parasternal short axis of the heart (Lekawanvijit *et al.* 2012). M-mode echocardiography was performed at the level of the papillary muscles. Percentage fractional shortening (%FS) and percentage left ventricular ejection fraction (%LVEF) were determined.

HRV analysis

The output from electrocardiogram (ECG) lead II was recorded in each rat using the PowerLab (ADInstruments, Sydney, NSW, Australia) and the Chart 5.0 programs (Raher *et al.* 2008). During ECG recording, rats were placed in a restraint and prohibited from movement (Incharoen *et al.* 2007, Raher *et al.* 2008, Kumfu *et al.* 2012). The high-frequency (HF, 0.6–3.0 Hz) component, representing cardiac parasympathetic activity, and low-frequency (LF, 0.2– 0.6 Hz) component, representing cardiac sympathetic and parasympathetic activity, were determined using the MATLAB program (Raher *et al.* 2008). The LF:HF ratio was considered to be an indicator of cardiac sympathetic/parasympathetic tone balance (Ohuchi *et al.* 2000). An increased LF:HF ratio (i.e. depressed HRV) indicated a cardiac sympathovagal imbalance (Incharoen *et al.* 2007).

Cardiac mitochondrial function determination

Cardiac mitochondrial isolation was performed as described previously (Thummasorn *et al.* 2011). Cardiac mitochondrial function was assessed by determining production of cardiac mitochondrial reactive oxygen species (ROS), cardiac mitochondrial membrane potential change, and cardiac mitochondrial swelling (Thummasorn *et al.* 2011, Apaijai *et al.* 2012, Chinda *et al.* 2013). The components of the buffers used in the analysis of cardiac mitochondrial function consisted of KCl, sucrose, HEPES, KH_2PO_4, and pyruvate/malate that have no ADP/ATP. The measurements of mitochondrial function in each group were made during State IV respiration.

Cardiac mitochondrial ROS production Cardiac mitochondria were incubated with 2-μM DCFH-DA dye at 25 °C for 20 min. The dye was excited at λ_{ex} 485 nm and detected at λ_{em} 530 nm using a fluorescent microplate reader (BioTek Instruments, Winooski, VT, USA). An increase in the fluorescence intensity indicated increased mitochondrial ROS production (Thummasorn *et al.* 2011, Apaijai *et al.* 2013, Chinda *et al.* 2013).

Cardiac mitochondrial membrane potential changes Cardiac mitochondrial membrane potential changes were determined using 5-μM JC-1 dye as described previously (Thummasorn *et al.* 2011, Apaijai *et al.* 2013). In brief, cardiac mitochondria were incubated with JC-1 at 37 °C for 30 min. JC-1 monomer form (green-fluorescent) was excited at λ_{ex} 485 nm and detected at λ_{em} 590 nm, and JC-1 aggregate form (red-fluorescent) was excited at λ_{ex} 485 nm and detected at λ_{em} 530 nm using a fluorescent microplate reader. A decrease in the red:green fluorescence intensity ratio indicated cardiac mitochondrial membrane depolarization (Thummasorn *et al.* 2011, Apaijai *et al.* 2013, Chinda *et al.* 2013).

Cardiac mitochondrial swelling Cardiac mitochondria were incubated with 1.5-mM respiration buffer containing 100 mM KCl, 10 mM HEPES, and 5 mM KH_2PO_4, and the absorbance was determined using a spectrophotometer as described previously (Thummasorn *et al.* 2011, Apaijai *et al.* 2013). Cardiac mitochondrial swelling was indicated by a decrease in the detected absorbance (Thummasorn *et al.* 2011, Apaijai *et al.* 2013, Chinda *et al.* 2013).

Cardiac mitochondrial morphology Cardiac mitochondria were fixed with 2.5% glutaraldehyde in 0.1-M

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phosphate buffer overnight and post-fixed in 1% cacodylate buffer osmium tetroxide for 2 h, and then dehydrated with a graded ethanol series (Thummasorn *et al.* 2011). Cardiac mitochondria were embedded in Epon-Araldite, cut with a diamond knife, and stained with uranyl acetate and lead acetate (Thummasorn *et al.* 2011). Cardiac mitochondrial morphology was detected using a transmission electron microscope (TEM; Thummasorn *et al.* 2011).

Western blot analysis of BAX and BCL2 protein expression levels

Myocardial protein extracts were prepared by homogenization of nitrogen-frozen myocardial tissues in a 300-ml extraction buffer containing 20-mM Tris–HCl (pH 6.8), 1-mM sodium orthovanadate, 5-mM sodium fluoride, and a protease inhibitor. Total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Samples of 50–80 μg of total protein were mixed with a loading buffer (5% betamercaptoethanol, 0.05% bromophenol blue, 75 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol), and loaded on 10% SDS–acrylamide gels. Proteins were transferred onto PVDF membrane in a glycine/methanol-transfer buffer (Palee *et al.* 2013, Surinkaew *et al.* 2013) in a Wet/Tank blotting system (Bio-Rad Laboratories). Membranes were blocked in 5% skim milk in Tris-buffered saline and Tween (TBST) buffer. Western blot analysis for BAX and BCL2 was performed using myocardial tissues. Membranes were exposed to mouse monoclonal anti-rat Bax and Bcl2 (1:1000 dilution, Santa Cruz Biotechnology). Bound antibody was detected by HRP conjugated with an anti-rabbit IgG (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA). The membranes were developed using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and densitometric analysis was carried out (Surinkaew *et al.* 2013).

Statistical analysis

Metabolic parameters, echocardiography, and HRV data are expressed as mean±S.D., and others are expressed as mean±S.E.M. One-way ANOVA followed by the least significant difference (LSD) *post hoc* test was used to test the differences among the groups. $P<0.05$ was considered statistically significant.

Results

Effects of pharmacological interventions on body weight, food intake, FBG, and plasma insulin in diabetic rats

At 10 days after STZ injection, diabetic rats were characterized by body weight loss, polyphagia, hyperglycemia, and insulin deficiency (Tables 1 and 2). Beginning at week 4 of insulin and combined drug treatments in diabetic rats, there were significantly increases in body weight and plasma insulin levels, and decreases in food intake and FBG levels, compared with DMV rats (Tables 3, 4 and 5). Beginning at week 4 for both doses of PCA treatment in diabetic rats, the FBG level significantly decreased when compared with DMV rats, whereas there was no alteration in body weight, food intake, and plasma insulin level in these rats (Tables 3, 4 and 5).

Table 1 Metabolic parameters, echocardiography, and heart rate variability (HRV) in normal and diabetic rats at baseline and 10 days after vehicle/STZ injection. Values are expressed as mean±S.D.

	Baseline		10 days after vehicle/STZ injection	
Parameters	NM	DM	NM	DM
Metabolic parameters				
Body weight (g)	274±9	274±11	316±9*	261±17†
Food intake (g/day)	20±2	21±2	22±2	24±2†
Fasting blood glucose (mg/dl)	94±3	95±6	100±5	400±36†
Plasma insulin (ng/ml)	1.49±0.37	1.48±0.38	1.39±0.44	0.56±0.25†
Plasma MDA (μmol/ml)	1.05±0.03	1.03±0.03	1.04±0.02	1.04±0.02
Echocardiography				
Fractional shortening (%)	55±3	54±4	55±2	53±3
LV ejection fraction (%)	81±3	82±3	81±2	75±4
HRV				
LF:HF ratio	0.24±0.04	0.24±0.04	0.21±0.04	0.23±0.04

NM, normal rat; DM, diabetic rat; LV ejection fraction, left ventricular ejection; LF:HF ratio, low-frequency:high-frequency ratio. *$P<0.05$ vs baseline and †$P<0.05$ vs NM at 10 days after vehicle injection.

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Table 2 Metabolic parameters, echocardiography, and heart rate variability (HRV) at 10 days after vehicle/STZ injection. Values are expressed as mean ± S.D. of five to six rats in each group

Parameters	NMV	DMV	DMI	DML	DMH	DMHI
Metabolic parameters						
Body weight (g)	316 ± 9	261 ± 14*	270 ± 20*	256 ± 12*	250 ± 16*	273 ± 16*
Food intake (g/day)	22 ± 2	24 ± 2*	24 ± 1*	24 ± 2*	25 ± 2*	25 ± 2*
Fasting blood glucose (mg/dl)	100 ± 5	394 ± 33*	400 ± 40*	392 ± 26*	414 ± 48*	407 ± 36*
Plasma insulin (ng/ml)	1.51 ± 0.60	0.67 ± 0.29*	0.55 ± 13*	0.52 ± 0.31*	0.56 ± 0.27*	0.50 ± 0.30*
Plasma MDA (μmol/ml)	1.04 ± 0.02	1.04 ± 0.02	1.03 ± 0.03	1.04 ± 0.02	1.03 ± 0.02	1.03 ± 0.02
Echocardiography						
Fractional shortening (%)	55 ± 2	53 ± 2	55 ± 4	52 ± 4	54 ± 5	52 ± 3
LV ejection fraction (%)	80 ± 2	77 ± 3	76 ± 4	75 ± 4	75 ± 4	74 ± 5
HRV						
LF:HF ratio	0.21 ± 0.04	0.22 ± 0.03	0.22 ± 0.05	0.23 ± 0.03	0.26 ± 0.05	0.23 ± 0.06

NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH + insulin, 4 U/kg; LV ejection fraction, left ventricular ejection fraction; LF:HF ratio, low-frequency:high-frequency ratio; *$P<0.05$ vs NMV.

Effects of pharmacological interventions on plasma MDA in diabetic rats

At 4 weeks after the STZ injection, plasma MDA levels were significantly increased in DMV rats when compared with NMV rats (Tables 3, 4 and 5). All treatments in diabetic rats significantly decreased the plasma MDA level, compared with DMV rats.

Effects of pharmacological interventions on echocardiography in diabetic rats

During week 4 after the STZ injection, diabetic rats developed cardiac contractile dysfunction that was characterized by significantly decreased %FS and %LVEF when compared with NMV rats (Tables 3, 4 and 5). Moreover, beginning at the fourth week for the insulin group and combined drug group, and in the eighth week after both doses of PCA treatment, a significant increase in the %FS and %LVEF occurred in diabetic rats when compared with DMV rats (Tables 3, 4 and 5). Interestingly, beginning at week 8 of PCA treatment at a dose of 100 mg/kg in diabetic rats, the echocardiography results did not differ from those for DMI rats. During week 12 of the combined drug treatment in diabetic rats, the echocardiography was restored to a normal status, whereas DMI alone could not achieve a similar benefit as %FS and %LVEF were still lower than those in the normal rats.

Effects of pharmacological interventions on HRV in diabetic rats

At 4 weeks after STZ injection, diabetic control rats had a significantly increased LF:HF ratio when compared with NMV rats, indicating a cardiac autonomic imbalance

Table 3 Effects of pharmacological interventions on metabolic parameters, echocardiography, and heart rate variability (HRV) at week 4 of treatment. Values are expressed as mean ± S.D. of five to six rats in each group

Parameters	NMV	DMV	DMI	DML	DMH	DMHI
Metabolic parameters						
Body weight (g)	397 ± 23	237 ± 40*	310 ± 18*,†	234 ± 25*,‡	241 ± 16*,‡	296 ± 30*,†
Food intake (g/day)	23 ± 2	34 ± 2*	29 ± 1*,†	33 ± 2*,‡	33 ± 2*,‡	29 ± 2*,†
Fasting blood glucose (mg/dl)	99 ± 5	431 ± 88*	166 ± 25*,†	316 ± 42*,†,‡	291 ± 58*,†,‡	155 ± 42*,†
Plasma insulin (ng/ml)	1.30 ± 0.29	0.32 ± 0.13*	1.50 ± 0.86†	0.57 ± 0.20*,‡	0.46 ± 0.09*,‡	1.28 ± 0.10†
Plasma MDA (μmol/ml)	1.03 ± 0.08	1.14 ± 0.10*	1.05 ± 0.05†	1.03 ± 0.04†	1.00 ± 0.02†	0.98 ± 0.06†
Echocardiography						
Fractional shortening (%)	58 ± 4	43 ± 3*	55 ± 3†	43 ± 2*,‡	44 ± 2*,‡	56 ± 2†
LV ejection fraction (%)	82 ± 3	68 ± 4*	79 ± 3†	68 ± 2*,‡	69 ± 3*,‡	80 ± 3†
HRV						
LF:HF ratio	0.23 ± 0.08	0.45 ± 0.06*	0.269 ± 0.025†	0.42 ± 0.04*,‡	0.41 ± 0.03*,‡	0.25 ± 0.06†

NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH + insulin, 4 U/kg; LV ejection fraction, left ventricular ejection fraction; LF:HF ratio, low-frequency:high-frequency ratio. *$P<0.05$ vs NMV, †$P<0.05$ vs DMV, and ‡$P<0.05$ vs DMI.

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Table 4 Effects of pharmacological interventions on metabolic parameters, echocardiography, and heart rate variability (HRV) at week 8 of treatment. Values are expressed as mean±S.D. of five to six rats in each group

Parameters	NMV	DMV	DMI	DML	DMH	DMHI
Metabolic parameters						
Body weight (g)	411±46	233±23*	323±32*,†	244±23*,‡	243±17*,‡	319±24*,†
Food intake (g/day)	24±1	36±1*	30±1*,†	34±1*,‡	35±2*,‡	31±2*,†
Fasting blood glucose (mg/dl)	94±6	441±42*	164±42*,†	331±46*,†,‡	270±53*,†,‡	156±31*,†
Plasma insulin (ng/ml)	1.71±1.14	0.43±0.14*	1.49±1.01†	0.42±0.08*,‡	0.46±0.19*,‡	2.01±0.91†
Plasma MDA (μmol/ml)	1.87±0.03	2.09±0.08*	1.88±0.03†	1.90±0.04†	1.89±0.03†	1.88±0.02†
Echocardiography						
Fractional shortening (%)	58±4	33±4*	55±5†	49±1*,†,‡	51±1*,†	55±4†
LV ejection fraction (%)	82±3	55±4*	79±4†	74±1*,†,‡	75±2*,†	80±3†
HRV						
LF:HF ratio	0.23±0.06	0.55±0.06*	0.31±0.10†	0.44±0.06*,†,‡	0.37±0.07*,†	0.30±0.06†

NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH+insulin, 4 U/kg; LV ejection fraction, left ventricular ejection fraction; LF:HF ratio, low-frequency/high-frequency ratio. *$P<0.05$ vs NMV, †$P<0.05$ vs DMV, and ‡$P<0.05$ vs DMI.

(Table 3). During the fourth week of insulin and of combined drug treatment, and week 8 of both dosages of PCA, there was a significant decrease in the LF:HF ratio in diabetic rats, compared with DMV rats (Tables 3, 4 and 5). Interestingly, beginning at week 8 of PCA treatment at a dose of 100 mg/kg in diabetic rats, the HRV did not differ from that for insulin treatment in diabetic rats.

Effects of pharmacological interventions on plasma HbA1c levels in diabetic rats

At week 12 of treatment, DMV had significantly elevated plasma HbA1c levels when compared with NMV rats. PCA (both doses), insulin, and combined drug treatments in diabetic rats decreased the plasma HbA1c levels, compared with DMV rats (Fig. 1A).

Effects of pharmacological interventions on cardiac MDA levels in diabetic rats

At the end of treatment, cardiac MDA levels were significantly increased in DMV rats, compared with NMV rats (Fig. 1B). PCA (both doses), insulin, and combined drug treatments in diabetic rats significantly decreased cardiac MDA levels, compared with DMV rats. Interestingly, in DMH, the cardiac MDA level decreased similarly to that of to DMI rats.

Effects of pharmacological interventions on cardiac mitochondrial function and morphology in diabetic rats

Cardiac mitochondrial ROS production at the end of treatment was significantly increased in DMV rats,

Table 5 Effects of pharmacological intervention on metabolic parameters, echocardiography, and heart rate variability (HRV) at week 12 of treatment. Values are expressed as mean±S.D. of five to six rats in each group

Parameters	NMV	DMV	DMI	DML	DMH	DMHI
Metabolic parameters						
Body weight (g)	444±23	225±12*	353±18*,†	243±21*,‡	246±19*,‡	339±23*,†
Food intake (g/day)	24±3	38±3*	33±2*,†	35±2*,‡	34±3*,‡	32±1*,†
Fasting blood glucose (mg/dl)	102±7	541±64*	184±26*,†	312±90*,†,‡	270±58*,†,‡	176±27*,†
Plasma insulin (ng/ml)	1.23±0.21	0.39±0.07*	1.39±0.81†	0.56±0.14*,‡	0.49±0.22*,‡	1.23±0.80†
Plasma MDA (μmol/ml)	2.14±0.02	2.25±0.05*	2.16±0.03†	2.19±0.03*,†	2.19±0.04*,†	2.13±0.02†
Echocardiography						
Fractional shortening (%)	57±3	33±3*	52±3*,†	49±3*,†	48±5*,†	56±3†,‡
LV ejection fraction (%)	82±2	54±5*	75±3*,†	74±3*,†	73±5*,†	80±3†,‡
HRV						
LF:HF ratio	0.24±0.05	0.54±0.13*	0.31±0.04†	0.41±0.06*,†	0.34±0.04*,†	0.29±0.14†

NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH+insulin, 4 U/kg; LV ejection fraction, left ventricular ejection fraction; LF:HF ratio, low-frequency:high-frequency ratio. *$P<0.05$ vs NMV, †$P<0.05$ vs DMV, and ‡$P<0.05$ vs DMI.

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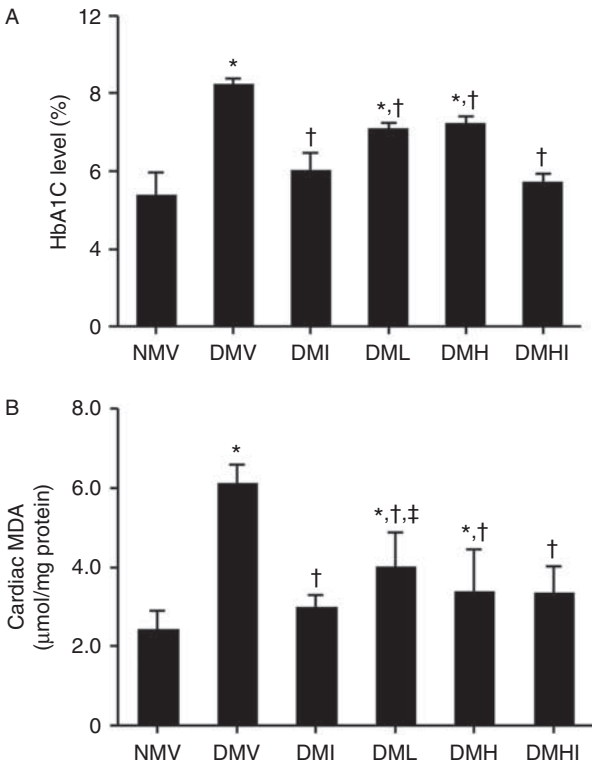

Figure 1
Effects of pharmacological interventions on plasma HbA1c and cardiac MDA levels. At the end of treatments, (A) plasma HbA1c level was increased in DMV rats, all treatments significantly decreased plasma HbA1c levels in diabetic rats. (B) Cardiac MDA level was elevated in DMV rats, and all treatments decreased cardiac MDA level in diabetic rats. Values are expressed as mean ± S.E.M. NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH + insulin, 4 U/kg. *$P<0.05$ vs NMV, †$P<0.05$ vs DMV, ‡$P<0.05$ vs DMI.

compared with NMV rats (Fig. 2A). PCA (both doses), insulin, and combined drug treatments in diabetic rats significantly reduced the mitochondrial ROS levels, compared with DMV rats. For mitochondrial membrane potential change, the red:green fluorescence intensity ratio was significantly decreased in DMV rats, compared with NMV rats, indicating cardiac mitochondrial membrane depolarization. All treatments attenuated mitochondrial depolarization, compared with DMV rats (Fig. 2B). For assessment of cardiac mitochondrial swelling, the absorbance was significantly decreased in the DMV rats, compared with NMV rats, indicating mitochondrial swelling (Fig. 2C). Moreover, PCA (both doses), insulin, and combined drug treatments significantly decreased mitochondrial swelling in diabetic rats, compared with DMV rats (Fig. 2C). The TEM images of cardiac mitochondria from each group are shown in Fig. 2D. Compared with the intact cardiac mitochondria of NMV rats, unfolded cristae were mostly found in DMV rats, indicating mitochondrial swelling. All treatments attenuated mitochondrial swelling, as indicated by decreased unfolded cristae in diabetic rats.

Effects of pharmacological interventions on BAX and BCL2 protein expression in the heart of diabetic rats

At week 12 after treatment, in the DMV rats, although BAX expression was not changed (Fig. 3A), BCL2 expression was significantly decreased compared with NMV rats (Fig. 3B). PCA (both doses), insulin, and combined drug treatments significantly increased BCL2 protein levels in diabetic rats, compared with DMV rats (Fig. 3B and C).

Discussion

This study demonstrated that STZ-induced type 1 diabetic rats were characterized by hyperglycemia, insulin deficiency, body weight loss, polyphagia, increased oxidative damage, depressed HRV, cardiac contractile dysfunction, cardiac mitochondrial dysfunction, and decreased cardiac anti-apoptotic BCL2 protein levels. The insulin and combined drug treatments increased the plasma insulin level, elevated body weight, and reduced food intake. In addition, all treatments in diabetic rats decreased the blood glucose level, reduced oxidative damage, improved HRV, attenuated cardiac dysfunction, prevented cardiac mitochondrial dysfunction, and increased BCL2 protein expression in diabetic rats. Interestingly, PCA at a dose of 100 mg/kg improved HRV and cardiac dysfunction similarly to insulin treatment in diabetic rats. Moreover, diabetic rats treated with combined drugs had echocardiography results restored to a normal status, whereas insulin alone could not achieve this. Insulin and combined drug treatments in diabetic rats also restored HRV.

A previous study demonstrated that PCA administration prevented the increase in plasma glucose and HbA1c levels and the decrease in plasma insulin levels in STZ-induced diabetic rats (Harini & Pugalendi 2010). PCA normalized the activities of gluconeogenic enzymes such as glucose 6-phosphatase and fructose 1,6-bisphosphatase, as well as that of the glycolytic enzyme glucokinase (Harini & Pugalendi 2010). In STZ-induced diabetic mice, dietary supplementation with PCA improved glycemic control and attenuated homeostatic disorders (Lin *et al.* 2009, 2011). Consistent with our findings, PCA treatment alone decreased hyperglycemia, whereas it could not

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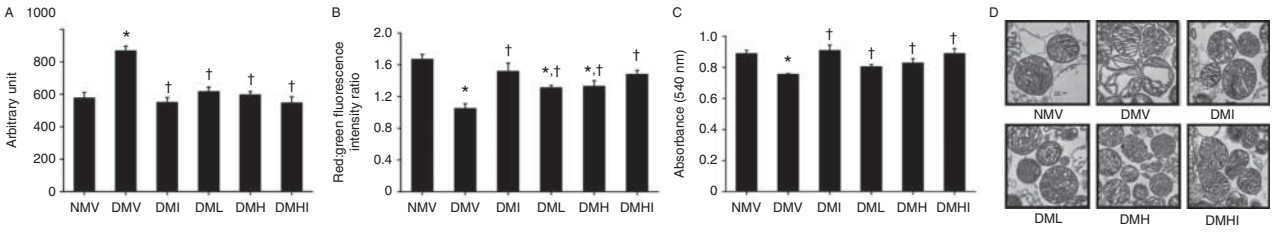

Figure 2

Effects of pharmacological interventions on cardiac mitochondrial function and morphology. (A) Cardiac mitochondrial ROS production was increased in DMV rats; all treatments reduced mitochondrial ROS production in diabetic rats. (B) Cardiac mitochondrial swelling was increased in DMV rats; all treatments prevented mitochondrial swelling in diabetic rats. (C) Cardiac mitochondrial membrane depolarization was increased in DMV rats; all treatments prevented mitochondrial membrane depolarization in diabetic rats. (D) Representative TEM images of mitochondrial morphology. Values are expressed as mean ± S.E.M. NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH + insulin, 4 U/kg. $^{*}P<0.05$ vs NMV and $^{\dagger}P<0.05$ vs DMV.

increase insulin levels in STZ-induced diabetic rats. As PCA has been shown to exert an insulin-like activity by increasing glucose uptake via enhancing GLUT4 translocation and adiponectin secretion caused by the increased PPARγ activity in adipocytes (Scazzocchio *et al*. 2011), this could be the mechanism responsible for its glycemic effect observed in this study.

The increased ROS generation and impaired antioxidant defenses both contribute to oxidative stress in diabetes. Most of the ROS generated within cells are from mitochondria that were exposed to hyperglycemia (Brownlee 1995, Shen *et al*. 2006). The MDA is an index of oxidative damage. Results from previous studies indicated that plasma and tissue MDA levels are elevated in STZ-induced diabetic rats, and that PCA decreased plasma, renal, and cardiac MDA levels, as well as elevated antioxidant defense in STZ-induced diabetic mice (Lin *et al*. 2009, Naowaboot *et al*. 2009). Consistent with results from our study, diabetic rats treated with PCA had decreased oxidative damage in both plasma and cardiac tissues. PCA also attenuated cardiac mitochondrial ROS production in diabetic rats, especially PCA at a dose of 100 mg/kg, which exhibited a strong potency similar to that of the insulin treatment. Increased ROS has been proposed to amplify hyperglycemia-induced MAPK isoform activation and increase advanced glycation end product (AGE) formation (Brownlee 1995, Koya & King 1998), which could promote cell injury that contributes to the development of cardiac dysfunction in diabetes. HbA1c is one of the AGEs, a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. Monitoring HbA1c in type 1 diabetic patients may improve the outcomes (Larsen *et al*. 1990). Results from a previous study indicated that PCA decreased plasma HbA1c levels and tissue AGEs in STZ-induced diabetic mice (Lin *et al*. 2011). Consistent with our findings, PCA decreased plasma HbA1c levels in STZ-induced diabetic rats.

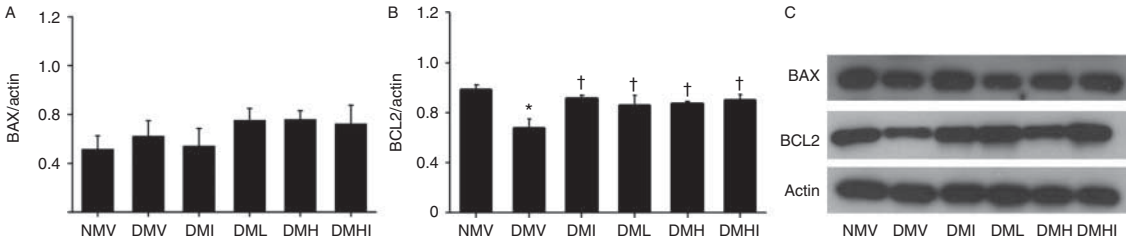

Figure 3

Effects of pharmacological interventions on cardiac BAX and BCL2 levels. (A) Cardiac BAX expression was not different among groups. (B) Cardiac BCL2 expression was decreased in DMV rats; all treatments increased cardiac BCL2 expression in diabetic rats. (C) Representative blot bands for BAX and BCL2 in each group. Values are expressed as mean ± S.E.M. NMV, normal rats treated with vehicle; DMV, diabetic rats treated with vehicle; DMI, diabetic rats treated with insulin, 4 U/kg; DML, diabetic rats treated with PCA, 50 mg/kg; DMH, diabetic rats treated with PCA, 100 mg/kg; DMHI, DMH + insulin, 4 U/kg. $^{*}P<0.05$ vs NMV and $^{\dagger}P<0.05$ vs DMV.

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In human studies, progressive autonomic dysfunction and depressed HRV in diabetic patients indicated cardiac autonomic neuropathy (Malpas & Maling 1990, Kudat *et al.* 2006). The results from an animal study indicated that HRV was depressed in the STZ-induced diabetic rat (Fazan *et al.* 1997). As depressed HRV has been associated with increased oxidative damage, such as increased plasma MDA levels (Pavithran *et al.* 2008), the prevention of depressed HRV in diabetic rats by PCA could be due to its ability to decrease oxidative stress in this study.

Results from previous studies indicated that the cardiac function index including ejection fraction and stroke volume was depressed at 4 weeks following STZ-induced diabetes (Crespo *et al.* 2008, 2011). Consistent with our findings, echocardiography showed a reduction of %FS and %LVEF in diabetic rats in this study. Moreover, mitochondria are known to be the source of energy required for the heart to function properly. The effects of PCA in reducing oxidative stress as well as attenuating cardiac mitochondrial dysfunction could be responsible for improved cardiac function as observed in this study. Supporting this statement is the fact that beginning at 4 weeks for insulin or combined drug treatments and at 8 weeks for both doses of PCA treatment, improved cardiac function was observed in STZ-induced diabetic rats included in our study. It is important to note that despite the fact that PCA improved cardiac function, insulin could provide this improvement faster (by the fourth week of treatment) than PCA (by the eighth week).

Overproduction of ROS in diabetes can activate the mitochondrial pathway, resulting in loss of mitochondrial membrane integrity and release of cytochrome *c*, apoptotic protease-activating factor 1, and other pro-apoptotic factors in the cytoplasm. Maintenance of mitochondrial membrane potential depends on pro-apoptotic (Bax) and anti-apoptotic (Bcl2) members of Bcl2 family, causing or preventing cytochrome *c* release. PCA has been shown to prevent apoptosis by attenuating the change in the mitochondrial membrane permeability, decreasing oxidative damage, and increasing anti-apoptotic BCL2 expression in MPP^+-induced mitochondrial dysfunction and apoptotic cell death in PC12 cells (Guan *et al.* 2006). These findings were consistent with our study demonstrating that PCA improved cardiac mitochondrial function and increased cardiac BCL2 level in diabetic rats.

In summary, in the hearts of STZ-induced diabetic rats, cardiac autonomic imbalance and cardiac dysfunction were developed. PCA, insulin, and combined drug treatments attenuated these adverse effects by attenuating hyperglycemia, oxidative damage, cardiac autonomic imbalance, and cardiac mitochondrial dysfunction, and increasing anti-apoptotic protein. These findings indicated that PCA could exert beneficial effects on the heart in type 1 DM and may be used as a supplement to prevent cardiac complications in diabetic patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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