British Journal of Pharmacology (2005) 145, 767–774
www.nature.com/bjp

Pomegranate flower improves cardiac lipid metabolism in a diabetic rat model: role of lowering circulating lipids

[1]Tom Hsun-Wei Huang, [1]Gang Peng, [1]Bhavani Prasad Kota, [1]George Qian Li, [2]Johji Yamahara, [1]Basil D. Roufogalis & *,[1]Yuhao Li

[1]Herbal Medicines Research and Education Centre, Faculty of Pharmacy, The University of Sydney, NSW, 2006, Australia and [2]Pharmafood Institute, Kyoto, Japan

1 Excess triglyceride (TG) accumulation and increased fatty acid (FA) oxidation in the diabetic heart contribute to cardiac dysfunction. *Punica granatum* flower (PGF) is a traditional antidiabetic medicine. Here, we investigated the effects and mechanisms of action of PGF extract on abnormal cardiac lipid metabolism both *in vivo and in vitro*.

2 Long-term oral administration of PGF extract (500 mg kg^{-1}) reduced cardiac TG content, accompanied by a decrease in plasma levels of TG and total cholesterol in Zucker diabetic fatty (ZDF) rats, indicating improvement by PGF extract of abnormal cardiac TG accumulation and hyperlipidemia in this diabetic model.

3 Treatment of ZDF rats with PGF extract lowered plasma FA levels. Furthermore, the treatment suppressed cardiac overexpression of mRNAs encoding for FA transport protein, peroxisome proliferator-activated receptor (PPAR)-α, carnitine palmitoyltransferase-1, acyl-CoA oxidase and 5′-AMP-activated protein kinase α2, and restored downregulated cardiac acetyl-CoA carboxylase mRNA expression in ZDF rats, whereas it showed little effect in Zucker lean rats. The results suggest that PGF extract inhibits increased cardiac FA uptake and oxidation in the diabetic condition.

4 PGF extract and its component oleanolic acid enhanced PPAR-α luciferase reporter gene activity in human embryonic kidney 293 cells, and this effect was completely suppressed by a selective PPAR-α antagonist MK-886, consistent with the presence of PPAR-α activator activity in the extract and this component.

5 Our findings suggest that PGF extract improves abnormal cardiac lipid metabolism in ZDF rats by activating PPAR-α and thereby lowering circulating lipid and inhibiting its cardiac uptake.
British Journal of Pharmacology (2005) **145,** 767–774. doi:10.1038/sj.bjp.0706245;
published online 9 May 2005

Keywords: *Punica granatum*; PPAR-α; lipid metabolism; myocardium; diabetes; fatty acid; oxidation; gene

Abbreviations: ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; AMPK, 5′-AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase1; FA, fatty acid; FATP, fatty acid transport protein; HEK, human embryonic kidney; NEFA, nonesterified free fatty acid; PPAR-α, peroxisome proliferator-activated receptor-α; PGF, *Punica granatum* flowers; RT–PCR, reverse transcriptase polymerase chain reaction; TC, total cholesterol; TG, triglyceride; ZDF, Zucker diabetic fatty; ZL, Zucker lean

Introduction

To meet the high-energy demands of the contracting muscle, the heart produces a constant and available supply of ATP. This energy is primarily produced by the metabolism of fatty acids (FAs) and carbohydrates. FA oxidation normally provides 60–70% of the ATP production by the heart, depending to a large extent on circulating FA concentrations. FAs are not as efficient as glucose as a source of myocardial energy (with respect to oxygen consumption), and require approximately 10% more oxygen to produce the equivalent amount of ATP. It has been shown recently that there is a switch in cardiac energy metabolism in diabetic patients (reviewed by Lopaschuk, 2002). In diabetes, in which plasma levels of both free FA and triglyceride (TG)-rich lipoproteins are increased, FA inhibition of both glycolysis and glucose oxidation in the heart is especially prominent. In uncontrolled diabetes, FA oxidation can provide from 90 to 100% of the heart's ATP requirements. Increased FA uptake and oxidation in the myocardium contribute to cardiac dysfunction, including congestive heart failure (Lopaschuk, 2002). On the other hand, TG accumulation in the heart is important for development of diabetic cardiomyopathy in mice (Nielsen *et al*., 2002). Hearts in *ob*/*ob* mice showed marked accumulation of neutral lipid droplets, which is paralleled by cardiac diastolic dysfunction (Christoffersen *et al*., 2003). Zucker diabetic fatty (ZDF) rats (a genetic animal model for type II diabetes and obesity) have markedly increased cardiac TG accumulation (Zhou *et al*., 2000), which induces lipotoxicity, thereby predisposing the myocytes to death and contractile dysfunction (Zhou *et al*., 2000).

Numerous herbs are available for the treatment of diabetes. Unfortunately, little is known about their effects on cardiac lipid metabolism. *Punica granatum* (PG) Linn., commonly known as pomegranate, is a small tree, belonging to the

*Author for correspondence; E-mail: yuhao@pharm.usyd.edu.au

Punicaceae family. Pomegranate juice and wine have become increasingly popular because of the attribution to them of important biological actions (Gil *et al*., 2000; Schubert *et al*., 2002), including cardiovascular protection (Aviram *et al*., 2002). Pomegranate juice has recently been demonstrated to improve lipid profiles in type II diabetic patients with hyperlipidemia (Esmaillzadeh *et al*., 2004). PG flowers (PGF) have been prescribed in *Unani* and *Ayurvedic* medicines for the treatment of diabetes (Jurjani, 1878; Majoosi, 1889). It has been demonstrated that PGF extract shows hypoglycemic activity in normal and alloxan-induced diabetic animals (Jafri *et al*., 2000). Recently, we have demonstrated that PGF extract improves postprandial hyperglycemia (Li *et al*., 2005) and glucose tolerance (Huang *et al*., 2005). Little is known, however, about regulation of lipid metabolism by pomegranate and PGF.

In the present study, we have investigated the effects and mechanisms of action of PGF extract on abnormalities of cardiac lipid metabolism in ZDF rats and its activities in a cell line.

Methods

Materials

Gallic acid (GA), fenofibrate, phorbol 12-myristate 13-acetate (PMA), oleanolic acid (OA) and ursolic acid (UA) were obtained from Sigma (Australia). MK-886 was purchased from Biomol (U.S.A.). The kits for determination of total cholesterol (TC), TG and nonesterified free FA (NEFA) contents were purchased commercially (Wako, Osaka, Japan).

All animal experimental procedures have been approved by the Animal Ethics Committee of the University of Sydney, Australia. Male Zucker lean (ZL) (fa/?) and ZDF (fa/fa) rats aged 13–15 weeks (Monash University Animal Services, Victoria, Australia) were housed in an air-conditioned room at $23 \pm 1^{\circ}$C with a 12-h light/dark cycle and were provided with standard food and water *ad libitum*. Animals were allowed free access to standard food and water for 1 week before starting the experiments.

Preparation of PGF extract

PGF was collected in June 2002 in Maharashtra state, India. Dried PG flowers were extracted with methanol at room temperature three times with 5 volumes of methanol (W/V). The solvent was evaporated under reduced pressure below 50°C to give a methanolic extract (yield: 40%).

Extract administration and blood biochemical measurements

Plasma levels of TC, TG and NEFA were determined under nonfasted conditions before treatment (Week 0) using commercially available kits. Animals were divided into ZL control, ZL PGF, ZDF control and ZDF PGF groups (five animals per group). Test sample (500 mg kg^{-1}, suspended in 5% acacia) or vehicle was given orally by gavage once daily for 6 weeks. Plasma levels of TC, TG and NEFA were determined again at Week 4 under nonfasted condition and at Week 5 under fasted conditions.

Measurement of left ventricular TC and TG contents

After rats were killed under halothane anesthesia (nonfasted conditions) at Week 6, the hearts were rapidly excised, and the left ventricle was frozen in liquid nitrogen and stored at -80°C for the study of lipid measurement and mRNA analysis. One part of the left ventricle was homogenized and extracted with isopropanol (1 ml/50 mg) (Oakes *et al*., 2001). After centrifugation, 5 μl aliquots of supernatant were added to 300 μl of reagent for enzymatic colorimetric determination of TC and TG contents (as above).

Tissue culture

Human embryonic kidney (HEK) 293 cell line was obtained from American Type Culture Collection. The cells were grown in DMEM/F-12, as previously described (Bramlett *et al*., 2003).

Gene expression analysis

Total mRNA was prepared separately from the left ventricle of individual rats using TRIzol (Invitrogen, Australia). The relative levels of specific mRNAs were assessed by reverse transcriptase polymerase chain reaction (RT–PCR), as described previously (Abe *et al*., 2002). Single-stranded cDNA was synthesized from 1 μg of total RNA using SuperScript II Rnase H Reverse Transcriptase, as per instructions of the manufacturer (Invitrogen, Australia). PCR was performed on a thermocycler, PTC-200 DNA engine (MJ Research Inc., MA, U.S.A.). The required cDNA was synthesized with the Platinum® Pfx DNA Polymerase method (Invitrogen, Australia). The genes examined were FA transport protein (FATP), peroxisome proliferator-activated receptor (PPAR)-α, carnitine palmitoyltransferase (CPT)-1, acyl-CoA oxidase (ACO), 5′-AMP-activated protein kinase (AMPK) α2 and acetyl-CoA carboxylase (ACC) from the left ventricle. The sequences of the sense and antisense primers used for amplification can be found in Table 1. The PCR samples were electrophoresed on 3% agarose gels and stained with ethidium bromide. The gel images were digitally captured with a CCD camera and analyzed with the ImageJ $\times$1.29 (NIH, U.S.A.). RT–PCR values are presented as a ratio of the specified gene signal in the selected linear amplification cycle divided by the β-actin signal.

Transfection and luciferase assay

At 48 h before transfection, HEK293 cell line was seeded at 5×10^5 cells /T25 flask in 5 ml of Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and supplemented with 1% penicillin and streptomycin, 1% L-glutamine, and 20 mM HEPES (Bramlett *et al*., 2003; Frederiksen *et al*., 2004). The plasmids used for transfection were tK-PPREx3-Luc plasmid (a kind gift from Dr Teruo Kawada, Kyoto University, Japan), pBI-G-hPPAR-α plasmid (a kind gift from Dr Sarah Roberts-Thomson, Queensland University, Australia) and pSV-β-Galactosidase Control Vector (Promega, Australia) to normalize transfection efficiencies. Cells were transfected with FuGENE 6 transfection reagent (Roche, Australia) in accordance with the manufacturer's instructions. After 24 h, cells were harvested and plated into 96-well plates

Table 1 Primers used in this study

Gene	*Accession number*	*Forward primer*	*Reverse primer*	*PCR product* (bp)	*Annealing temperature* (°C)
ACC	J03808	5′-AGGAGGGAAGGGAATCAGAA-3′	5′-TGTGCTGCAGGAAGATTGAC-3′	435	61
ACO	Zhou *et al*. (1998)	5′-GCCCTCAGCTATGGTATTAC-3′	5′-AGGAACTGCTCTCACAATGC-3′	634	61
AMPKα2	NM023991	5′-TATTGCCACTCTGCTGATGC-3′	5′-GGGCTGTCTGCTATGAGAGG-3′	414	63
CPT-1	AF029875	5′-GCAAACTGGACCGAGAAGAG-3′	5′-TCCATGAGGGATGGACTCTC-3′	496	63
FATP	U89529	5′-GGACCTTCGCACAGCTAGAC-3′	5′-AAATAGCCGATCATCCATGC-3′	425	64
PPAR-α	NM013196	5′-GACAAGGCCTCAGGATACCA-3′	5′-GTCTTCTCAGCCATGCACAA-3′	440	64
β-Actin	NM031144	5′-AGCCATGTACGTAGCCATCC-3′	5′-CTCTCAGCTGTGGTGGTGAA-3′	228	60

at 5×10^4 cells per well in complete transfection media and allowed to attach for 2 h. The cells were then treated with fenofibrate (100 μM) and test samples (PGF; 10, 50, 100 μg ml^{-1}, and GA, OA and UA; 10, 30, 300 μM). In additional experiments, antagonist MK-886 (20 μM) was added 1 h before fenofibrate and the test samples. After 24 h, the cells were lysed and assayed for luciferase and β-galactosidase activities using the Bright-Glo Luciferase Assay System and Beta-Glo Assay System (Promega, Australia), respectively. The results were expressed as relative luciferase activity normalized with β-galactosidase signal (fold difference compared to negative control).

Data analysis

All results are expressed as means ± s.e.m. Data were analyzed by 1-factor analysis of variance (ANOVA). If a statistically significant effect was found, the Newman–Keuls test was performed to isolate the difference between the groups. *P*-values less than 0.05 ($P<0.05$) were considered as indicative of significance.

Results

Effects of PGF extract on cardiac TG contents, plasma levels of TC, TG and NEFA, and body and liver weights in rats

The ZDF control showed approximately two-fold higher TG accumulation in the heart than ZL control (Figure 1b), whereas no significant difference was observed in the cardiac TC content between the hearts of ZL and ZDF controls (Figure 1a), under nonfasted conditions. Also ZDF controls showed much higher plasma levels of TC (Figure 2), TG (Figure 3) and NEFA (Figure 4) at Weeks 0 and 4 under nonfasted conditions. Treatment with PGF extract

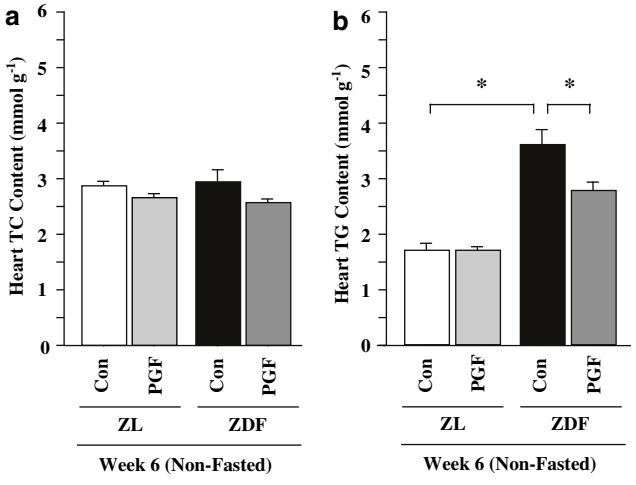

Figure 1 Excess cardiac triglyceride (TG) accumulation in Zucker diabetic fatty (ZDF) rats is improved by the extract of *Punica granatum* flowers (PGF). Zucker Lean (ZL) rats and ZDF rats were fed PGF extract (500 mg kg^{-1}) once daily for 6 weeks. Cardiac total cholesterol (TC) (a) and TG (b) contents were determined at Week 6. All values are means ± s.e.m. ($n=5$). *$P<0.05$. Con: control.

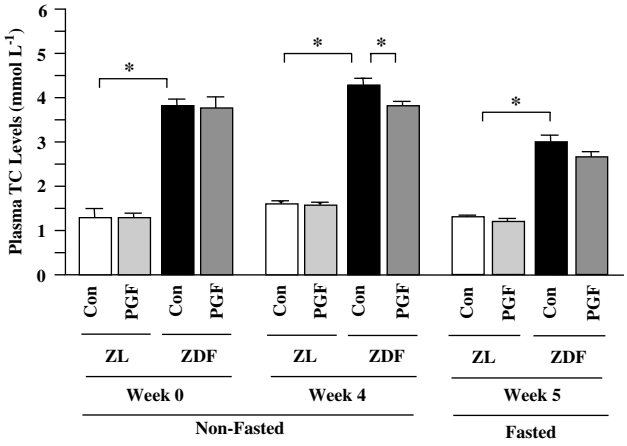

Figure 2 Hypercholesterolemia in ZDF rats is improved by PGF extract. ZL and ZDF rats were fed PGF extract (500 mg kg^{-1}) once daily. Plasma TC levels were determined before (Week 0) and 4 weeks (Week 4) after the treatment under nonfasted conditions. Plasma TC levels under fasting condition were determined again at Week 5. All values are means ± s.e.m. ($n = 5$). *$P < 0.05$. Con: control.

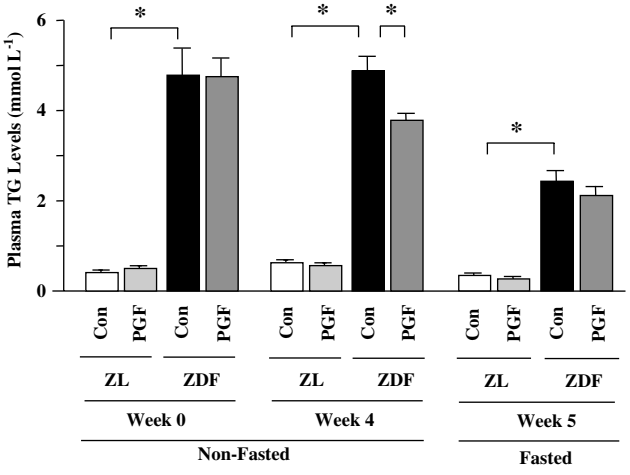

Figure 3 Hypertriglyceridemia in ZDF rats is improved by PGF extract. ZL and ZDF rats were fed PGF extract (500 mg kg^{-1}) once daily. Plasma TG levels were determined before (Week 0) and 4 weeks (Week 4) after the treatment under nonfasted conditions. Plasma TG levels under fasting condition were determined again at Week 5. All values are means ± s.e.m. ($n = 5$). *$P < 0.05$. Con: control.

(500 mg kg^{-1}) reduced cardiac TG content (Figure 1b) at Week 6, and plasma levels of TC (Figure 2), TG (Figure 3) and NEFA (Figure 4) at Week 4, under nonfasted conditions. Neither cardiac TC (Figure 1a) or TG (Figure 1b) contents, nor plasma TC (Figure 2a) or TG (Figure 2b) levels in ZL rats were affected by PGF extract.

18 h-fasting was more effective in reducing plasma levels of TC and TG in ZDF rats than ZL rats (Figures 2 and 3). The significant elevation in plasma TC and TG levels in ZDF control rats was lost in the ZDF PGF treatment group. In contrast, fasting markedly increased plasma NEFA levels in all groups (Figure 4). Treatment with PGF extract showed a

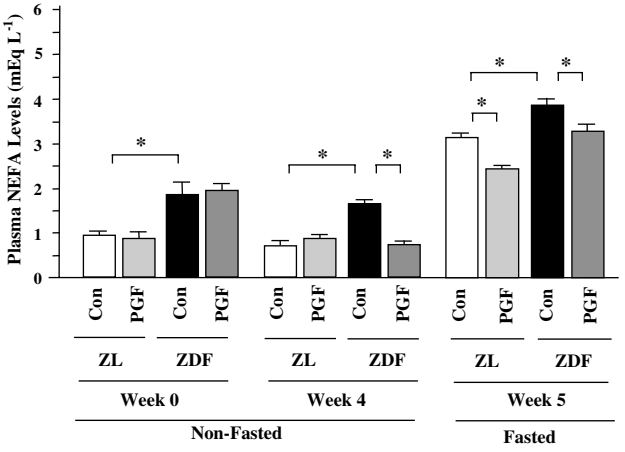

Figure 4 Effects of long-term administration of PGF extract on plasma nonesterified free fatty acid (NEFA) levels in ZL and ZDF rats. ZL and ZDF rats were fed PGF extract (500 mg kg^{-1}) once daily. Plasma NEFA levels were determined before (Week 0) and 4 weeks (Week 4) after the treatment under nonfasted conditions. Plasma NEFA levels under fasting condition were determined again at Week 5. All values are means ± s.e.m. ($n = 5$). *$P < 0.05$. Con: control.

significant inhibition of fasting plasma NEFA levels in both ZL and ZDF rats.

In all, 6-week administration of PGF extract did not significantly change body weight in ZL or ZDF rats (Week 6: ZL control 316.4 ± 10.5 g; ZL PGF 321.2 ± 12.3 g; ZDF control 426.4 ± 6.4 g; ZDF PGF 434.0 ± 15.2 g). In contrast, the treatment decreased the liver weight in ZDF rats, whereas it did not affect the liver weight in ZL rats (ZL Control 9.26 ± 0.38 g; ZL PGF 9.43 ± 0.46 g; ZDF Control 17.71 ± 0.42 g; ZDF PGF 15.99 ± 0.44 g).

Changes in gene expression profiles

To investigate the molecular mechanism of PGF extract-induced improvement of abnormal cardiac lipid metabolism, we examined the mRNA expression of cardiac lipogenic genes. The results showed that the expression of cardiac mRNAs encoding FATP (Figure 5a), PPAR-α (Figure 5b), CPT-1 (Figure 5c), ACO (Figure 5e) and AMPKα2 (Figure 5f) were upregulated, whereas ACC mRNA expression (Figure 5d) was downregulated, in the left ventricle of ZDF controls, compared to ZL controls. PGF extract treatment for 6 weeks reduced all abnormal cardiac mRNA expression in ZDF rats, whereas it showed little effect in ZL rats (Figure 5a–e).

Effects of PGF extract and its components on PPAR-α luciferase activity in cell lines

To further understand the mechanism of PGF extract in regulating lipid metabolism, we investigated the effects of PGF extract on PPAR-α luciferase activity in various *in vitro* experiments. The results showed that fenofibrate (a selective PPAR-α activator) (Yoshikawa *et al*., 2003) dose-dependently (100–300 μM) enhanced PPAR-α luciferase activity in HEK293 cell line transfected with PPAR-α reporter gene (Figure 6b).

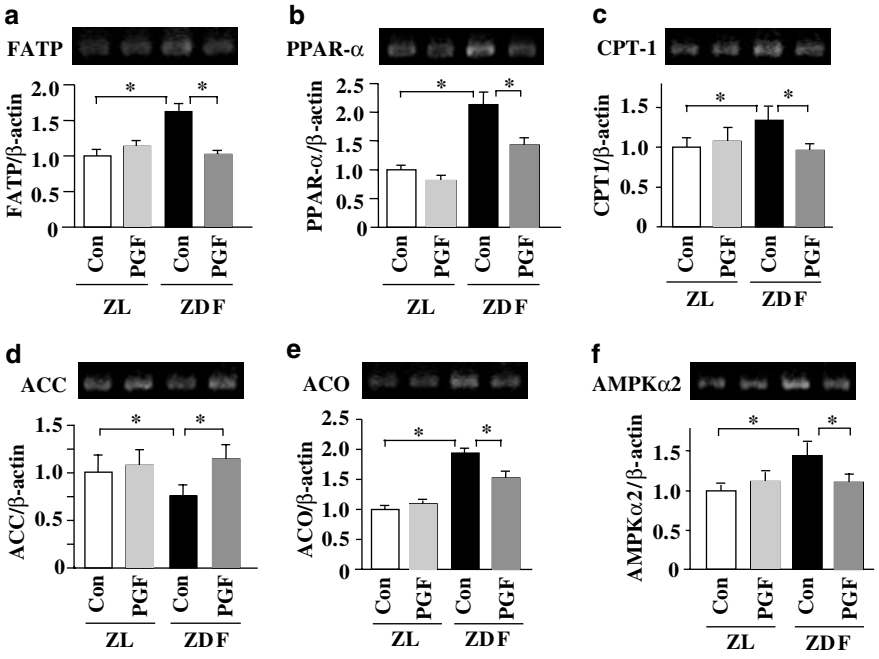

Figure 5 Changes in cardiac gene expressions in ZL and ZDF rats. Total mRNAs were prepared from the left ventricle using TRIzol. The relative levels of mRNAs encoding for (a) fatty acid transport protein (FATP), (b) carnitine palmitoyltransferase (CPT)-1, (c) peroxisome proliferator-activated receptor (PPAR)-α, (d) acetyl-CoA carboxylase (ACC), (e) acyl-CoA oxidase (ACO) and (f) AMPK, 5′-AMP–activated protein kinase (AMPKα2) were assessed by RT–PCR. Results were normalized to β-actin. Levels in ZL control were arbitrarily assigned a value of 1.0. All values are means ± s.e.m. ($n = 5$). *$P < 0.05$.

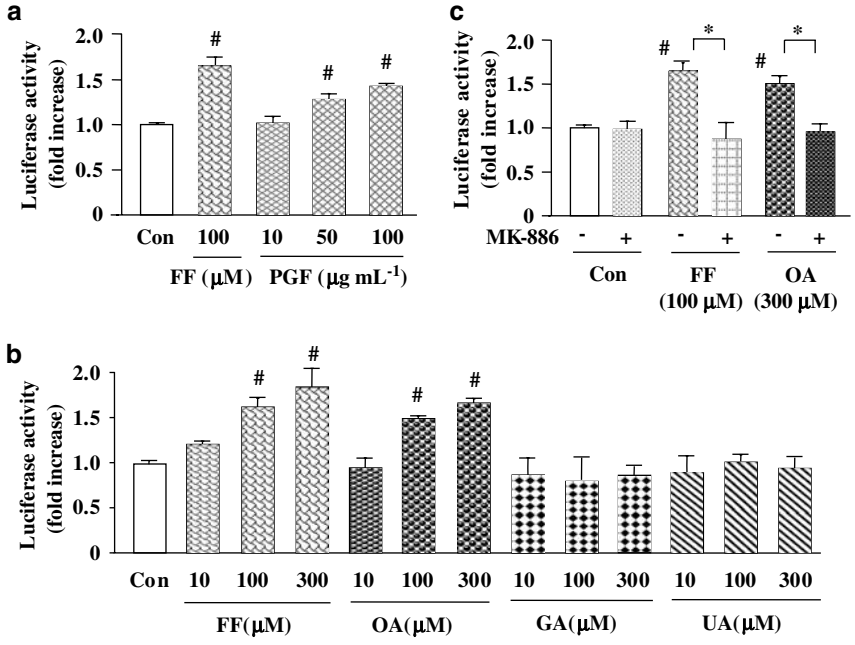

Figure 6 Effects of PGF extract and its component oleanolic acid (OA) on PPAR-α activity in human embryonic kidney (HEK293) cell line. The extract (a) and OA (b) dose-dependently enhance PPAR-α luciferase activity in HEK293 cells transfected with reporter genes. OA-induced enhancement of PPAR-α luciferase activity is completely suppressed by a selective PPAR-α antagonist MK-886 (20 μM) (c). Levels in control were arbitrarily assigned a value of 1.0. All values are means ± s.e.m. ($n = 3$, each in duplicate) vs control, # $P < 0.05$; *$P < 0.05$. FF: fenofibrate.

Similarly, PGF extract also concentration-dependently (10–100 $\mu g\,mL^{-1}$) enhanced PPAR-α luciferase activity (Figure 6a).

We recently demonstrated that PGF extract contains OA, UA and GA (our unpublished data). In order to identify active components, we tested the effects of OA, UA and GA on PPAR-α luciferase activity in the cell lines. The results showed that OA, but not UA and GA, concentration-dependently (100–300 μM) increased PPAR-α luciferase activity (Figure 6b).

OA (300 μM)-induced enhancement was completely suppressed by a selective PPAR-α antagonist MK-886 (Kehrer *et al*., 2001) (Figure 6c).

Discussion

One of the important findings in the present study is that oral administration of PGF extract improves excess cardiac TG accumulation, accompanied by improvement of hypertriglyceridemia and hypercholesterolemia in ZDF rats.

Since cardiac myocytes have little capacity for *de novo* biosynthesis or storage of long-chain FAs, the heart must import this metabolic substrate from the circulation. When myocardial FA level is elevated, unesterified FAs and acyl-CoAs that are not used for β-oxidation are sequestered into TG. Obesity and diabetes are conditions in which serum FAs and myocardial FA uptake are elevated (Stanley *et al*., 1997). In the present study, under nonfasting conditions PGF extract reduced plasma TG in ZDF rats but not in ZL control, possibly by reducing TG absorption and/or by decreasing formation of intestinal chylomicron particles. PGF extract reduced plasma NEFA levels in both nonfasting and fasting animals, suggesting reduction in FA production from TG hydrolysis and inhibition of adipose tissue lipolysis. The enhanced accumulation of TG in diabetic heart, and its reduction by PGF, may be accounted for by the reduced levels of plasma NEFA after PGF, with resulting reduced uptake of FA into the heart. It is well known that movement of long-chain FAs across the plasma membrane of mammalian cells is facilitated by FATP (Schaffer & Lodish, 1994). *Ob*/*ob* mouse hearts have increased expression of cardiac FATP and heart-specific FA binding protein mRNAs that stimulate myocyte FA uptake and TG storage and accumulate neutral lipids within the cardiac myocytes (Christoffersen *et al*., 2003). Atkinson *et al*. (2003) demonstrated that hearts from insulin-resistant rats (JCR:LA-cp rats) accumulate substantial amounts of TG as a result of increased FA supply. In the present study, ZDF rats also exhibited upregulated cardiac FATP mRNA expression in ZDF rats, which was suppressed by PGF extract. Thus, our results are consistent with the suggestion that PGF extract reduces cardiac TG by reducing FA supply following lowering of plasma FA levels and by inhibition of cardiac FATP expression.

In this study, we also found that ZDF rats overexpressed PPAR-α. The main effect of PPAR-α (a ligand-activated transcription factor activated by the binding of long-chain FA and related compounds; Escher & Wahli, 2000) in the heart is to provide energy to the myocardium by activating genes regulating mitochondrial FA uptake and oxidation (Vosper *et al*., 2002). Mice overexpressing PPAR-α in the heart show a cardiac phenotype mimicking that caused by diabetes, with enhancement of target genes involved in cardiac FA uptake and oxidation pathways (Finck *et al*., 2002). CPT-1 is a point for control and regulation of FA oxidation, which is indirectly controlled by ACC (Lopaschuk, 2002). An increase in AMPK activity closely correlates to a decrease in ACC activity and increased FA oxidation in isolated working rat hearts (Lopaschuk, 2002). Coort *et al*. (2004) demonstrated that the rate of palmitate oxidation was greater in cardiac myocytes from obese Zucker rats (a model without hyperglycemia) than ZL rats when they were stimulated by oligomycin (an AMPK activator and contraction-mimetic agent), although there was no difference in the basal condition between the two strains. A high capacity for FA oxidation is a hallmark of the diabetic myocardium (Rodrigues *et al*., 1998). Neitzel *et al*. (2003) demonstrated that rates of FA oxidation and esterification were increased in the hearts of db/db mice. More recently, Wang *et al*. (2004) have demonstrated that there was an increase in FA oxidation in isolated ZDF rats under all conditions of baseline perfusion, low flow ischemia, and following low flow ischemia and reperfusion. In the present study, we have shown that PGF extract attenuated the upregulation of cardiac mRNA expression of CPT-1 and AMPKα2 along with PPAR-α, and restored downregulated ACC mRNA in fed ZDF rats. These results suggest that PGF may improve excessive cardiac FA oxidation in ZDF rats.

A question arising from our current study is whether the action of PGF on the TG levels in the heart was more prominent through indirect pathways altering plasma FA levels or more directly through effects on cardiac PPAR-α and metabolic enzymes. PGF extract seemed to lower levels of plasma TG and NEFA, and cardiac FATP mRNA to a greater extent than those of cardiac CPT-1 and ACO mRNAs, suggesting that the predominant effect of PGF extract was to decrease cardiac FA uptake more than cardiac FA oxidation. Thus, the end result is a reduction of cardiac TG accumulation in ZDF rats. The fact that PGF extract-induced cardiac changes were seen in ZDF but not in corresponding lean rats, where plasma FA levels were also unchanged, also indicates that the action of PGF extract on the heart is predominantly indirect and secondary to reductions in plasma lipids. However, to further investigate the significance of the direct effects, it will be necessary to use rat primary cardiomyocytes, especially those from diabetic heart, or closely related cell lines. The potential consequence of cardiac TG lowering is to improve contractile function of diabetic hearts. It will be of interest to determine whether the effects of PGF extract on cardiac lipid metabolism, which we observed in ZDF rats, are accompanied by amelioration of abnormal cardiac function in diabetes and obesity, and to examine further the mechanisms of these actions.

PPAR-α agonists have been in use for over 40 years for the treatment of dyslipidemia, mainly due to their actions of lowering TG levels (Francis *et al*., 2003). Aasum *et al*. (2002) reported that chronic treatment of db/db mice with PPAR-α agonist normalized circulating FA and TG levels, and actually reduced myocardial FA oxidation by 50%. We speculated that PGF extract may have PPAR-α activator properties. To test this hypothesis, we investigated the effects of PGF extract on PPAR-α expression in various *in vitro* experiments. The results obtained demonstrated that PGF extract enhanced PPAR-α luciferase activity in HEK293 cell line transfected with PPAR-α reporter gene. Thus, our results suggest that the PPAR-α activator properties of PGF extract may be involved in the regulatory effect of PGF extract on lipid metabolism in ZDF rats.

OA (3β-hydroxy-olea-12-en-28-oic acid) and its isomer, UA (3β-hydroxy-urs-12-en-28-oic acid), are triterpenoid compounds that exist largely in food products (vegetable oils) (Perez-Camino & Cert, 1999), many of which are used as medicinal plants in traditional medicine. Liu (1995) have listed their main biopharmacological effects, including antidiabetogenic and antihyperlipidemic activities. GA (3,4,5-trihydrox-

ybenzoic acid) and its structurally related compounds are found widely distributed in fruits and plants. Studies utilizing these compounds have found them to possess many potential therapeutic properties, including anti-inflammatory effects (Kroes *et al*., 1992). Interestingly, our present results in cell lines demonstrated that of the components found in PGF extract OA, but not UA and GA, promote PPAR-α activation. Thus, OA could be, at least in part, responsible for PGF extract's cardiac metabolic improvement in diabetes and obesity.

Some synthetic PPAR-α agonists increase the liver weight in rodents (Larsen *et al*., 2003; Ameen *et al*., 2005). Interestingly, chronic administration of PGF extract did not increase but decreased the liver weight in ZDF rats, whereas it did not affect the liver weight in ZL rats. It has been reported that OA has hepatoprotective effects against chemical-induced hepatic injury (Liu *et al*., 1995; Jeong, 1999). OA has been marketed in China as an oral drug for human liver disorders (Liu, 1995). Although the hepatoprotective effect is proposed to account for a decrease of the liver weight in ZDF rats, the exact mechanism is still needs to be further clarified.

In conclusion, our findings show that PGF extract improves cardiac abnormalities of lipid metabolism in ZDF rats, and suggest that its property of activating PPAR-α and thereby lowering circulating lipids plays an important role in this action. These studies provide potentially important results, which may lead to further research supporting extension of the findings to clinical trials to demonstrate the effectiveness of PGF extract in the prevention and/or treatment of diabetes-related cardiovascular complications through modulation of abnormal lipid metabolism.

We thank Drs Andrew Cheung, Colin Duke, Van Hoan Tran and Mr Bruce Tattam for their suggestions and technical instructions in the phytochemistry.

References

AASUM, E., BELKE, D.D., SEVERSON, D.L., RIEMERSMA, R.A., COOPER, M., ANDREASSEN, M. & LARSEN, T.S. (2002). Cardiac function and metabolism in type 2 diabetic mice after treatment with BM 17.0744, a novel PPAR-alpha activator. *Am. J. Physiol. Heart Circ. Physiol.,* **283,** H949–H957.

ABE, A., KIRIYAMA, Y., HIRANO, M., MIURA, T., KAMIYA, H., HARASHIMA, H. & TOKUMITSU, Y. (2002). Troglitazone suppresses cell growth of KU812 cells independently of PPARgamma. *Eur. J. Pharmacol.,* **436,** 7–13.

AMEEN, C., EDVARDSSON, U., LJUNGBERG, A., ASP, L., AKERBLAD, P., TUNELD, A., OLOFSSON, S.O., LINDEN, D. & OSCARSSON, J. (2005). Activation of peroxisome proliferator-activated receptor alpha increases the expression and activity of microsomal triglyceride transfer protein in the liver. *J. Biol. Chem.,* **280,** 1224–1229.

ATKINSON, L.L., KOZAK, R., KELLY, S.E., ONAY BESIKCI, A., RUSSELL, J.C. & LOPASCHUK, G.D. (2003). Potential mechanisms and consequences of cardiac triacylglycerol accumulation in insulin-resistant rats. *Am. J. Physiol. Endocrinol. Metab.,* **284,** E923–E930.

AVIRAM, M., DORNFELD, L., KAPLAN, M., COLEMAN, R., GAITINI, D., NITECKI, S., HOFMAN, A., ROSENBLAT, M., VOLKOVA, N., PRESSER, D., ATTIAS, J., HAYEK, T. & FUHRMAN, B. (2002). Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: studies in atherosclerotic mice and in humans. *Drugs. Exp. Clin. Res.,* **28,** 49–62.

BRAMLETT, K.S., HOUCK, K.A., BORCHERT, K.M., DOWLESS, M.S., KULANTHAIVEL, P., ZHANG, Y., BEYER, T.P., SCHMIDT, R., THOMAS, J.S., MICHAEL, L.F., BARR, R., MONTROSE, C., EACHO, P.I., CAO, G. & BURRIS, T.P. (2003). A natural product ligand of the oxysterol receptor, liver X receptor. *J. Pharmacol. Exp. Ther.,* **307,** 291–296.

CHRISTOFFERSEN, C., BOLLANO, E., LINDEGAARD, M.L., BARTELS, E.D., GOETZE, J.P., ANDERSEN, C.B. & NIELSEN, L.B. (2003). Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology,* **144,** 3483–3490.

COORT, S.L., HASSELBAINK, D.M., KOONEN, D.P., WILLEMS, J., COUMANS, W.A., CHABOWSKI, A., VAN DER VUSSE, G.J., BONEN, A., GLATZ, J.F. & LUIKEN, J.J. (2004). Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese zucker rats. *Diabetes,* **53,** 1655–1663.

ESCHER, P. & WAHLI, W. (2000). Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutat Res.,* **448,** 121–138.

ESMAILLZADEH, A., TAHBAZ, F., GAIENI, I., ALAVI-MAJD, H. & AZADBAKHT, L. (2004). Concentrated pomegranate juice improves lipid profiles in diabetic patients with hyperlipidemia. *J. Med. Food,* **7,** 305–308.

FINCK, B.N., LEHMAN, J.J., LEONE, T.C., WELCH, M.J., BENNETT, M.J., KOVACS, A., HAN, X., GROSS, R.W., KOZAK, R., LOPASCHUK, G.D. & KELLY, D.P. (2002). The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. *J. Clin. Invest.,* **109,** 121–130.

FRANCIS, G.A., ANNICOTTE, J.S. & AUWERX, J. (2003). PPAR-alpha effects on the heart and other vascular tissues. *Am. J. Physiol. Heart. Circ. Physiol.,* **285,** H1–H9.

FREDERIKSEN, K.S., WULFF, E.M., SAUERBERG, P., MOGENSEN, J.P., JEPPESEN, L. & FLECKNER, J. (2004). Prediction of PPAR-alpha ligand-mediated physiological changes using gene expression profiles. *J. Lipid Res.,* **45,** 592–601.

GIL, M.I., TOMAS-BARBERAN, F.A., HESS-PIERCE, B., HOLCROFT, D.M. & KADER, A.A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food. Chem.,* **48,** 4581–4589.

HUANG, T.H., PENG, G., KOTA, B.P., LI, Q., YAMAHARA, J., ROUFOGALIS, BD. & LI, Y. (2005). Anti-diabetic action of *Punica granatum* flower extract: activation of PPAR-γ and identification of an active component. *Toxcol. Appl. Pharm.,* in press.

JAFRI, M.A., ASLAM, M., JAVED, K. & SINGH, S. (2000). Effect of *Punica granatum* Linn., (flowers) on blood glucose level in normal and alloxan-induced diabetic rats. *J. Ethnopharmacol.,* **70,** 309–314.

JEONG, H.G. (1999). Inhibition of cytochrome *P*450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. *Toxicol. Lett.,* **105,** 215–222.

JURJANI, M.I. (1878). *Zakheera-Khwarzam-Shahi*. Lucknow: Munshi Nawal Kishore. 540 (Urdu translation).

KEHRER, J.P., BISWAL, S.S., LA, E., THUILLIER, P., DATTA, K., FISCHER, S.M. & VANDEN HEUVEL, J.P. (2001). Inhibition of peroxisome-proliferator-activated receptor (PPAR) alpha by MK886. *Biochem. J.,* **356,** 899–906.

KROES, B.H., VAN DEN BERG, A.J., QUARLES VAN UFFORD, H.C., VAN DIJK, H. & LABADIE, R.P. (1992). Anti-inflammatory activity of gallic acid. *Planta Med.,* **58,** 499–504.

LARSEN, P.J., JENSEN, P.B., SORENSEN, R.V., LARSEN, L.K., VRANG, N., WULFF, E.M. & WASSERMANN, K. (2003). Differential influences of peroxisome proliferator-activated receptors gamma and -alpha on food intake and energy homeostasis. *Diabetes,* **52,** 224922–224959.

LI, Y., WEN, S., KOTA, B.P., PENG, G., LI, Q., YAMAHARA, J.& ROUFOGALIS, B.D. (2005). *Punica granatum* flower extract, a potent α-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. *J. Ethnopharmacol.,* in press.

LIU, J. (1995). Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.,* **49,** 57–68.

LIU, J., LIU, Y., PARKINSON, A. & KLAASSEN, C.D. (1995). Effect of oleanolic acid on hepatic toxicant-activating and detoxifying systems in mice. *J. Pharmacol. Exp. Ther.,* **275,** 768–774.

LOPASCHUK, G.D. (2002). Metabolic abnormalities in the diabetic heart. *Heart. Fail. Rev.,* **7,** 149–159.

MAJOOSI, A.I.A. (1889). *Kamilussanah*. Lucknow: Munshi Nawal Kishore. 145 Urdu translation.

NEITZEL, A.S., CARLEY, A.N. & SEVERSON, D.L. (2003). Chylomicron and palmitate metabolism by perfused hearts from diabetic mice. *Am. J. Physiol. Endocrinol. Metab.,* **284,** E357–E365.

NIELSEN, L.B., BARTELS, E.D. & BOLLANO, E. (2002). Overexpression of apolipoprotein B in the heart impedes cardiac triglyceride accumulation and development of cardiac dysfunction in diabetic mice. *J. Biol. Chem.,* **277,** 27014–27020.

OAKES, N.D., THALEN, P.G., JACINTO, S.M. & LJUNG, B. (2001). Thiazolidinediones increase plasma-adipose tissue FFA exchange capacity and enhance insulin-mediated control of systemic FFA availability. *Diabetes,* **50,** 1158–1165.

PEREZ-CAMINO, M.C. & CERT, A. (1999). Quantitative determination of hydroxy pentacyclic triterpene acids in vegetable oils. *J. Agric. Food. Chem.,* **47,** 1558–1562.

RODRIGUES, B., CAM, M.C. & MCNEILL, J.H. (1998). Metabolic disturbances in diabetic cardiomyopathy. *Mol. Cell Biochem.,* **180,** 53–57.

SCHAFFER, J.E. & LODISH, H.F. (1994). Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell,* **79,** 427–436.

SCHUBERT, S.Y., NEEMAN, I. & RESNICK, N. (2002). A novel mechanism for the inhibition of NF-kappaB activation in vascular endothelial cells by natural antioxidants. *FASEB J.,* **16,** 1931–1933.

STANLEY, W.C., LOPASCHUK, G.D. & MCCORMACK, J.G. (1997). Regulation of energy substrate metabolism in the diabetic heart. *Cardiovasc. Res.,* **34,** 25–33.

VOSPER, H., KHOUDOLI, G.A., GRAHAM, T.L. & PALMER, C.N. (2002). Peroxisome proliferator-activated receptor agonists, hyperlipidaemia, and atherosclerosis. *Pharmacol. Ther.,* **95,** 47–62.

WANG, P.G., LLOYD, S.G., ZENG, H., BONEN, A. & CHATHAM, J.C. (2004). The impact of altered substrate utilization on cardiac function in isolated hearts from Zucker diabetic fatty rats. *Am. J. Physiol. Heart. Circ. Physiol.,* in press.

YOSHIKAWA, T., IDE, T., SHIMANO, H., YAHAGI, N., AMEMIYA-KUDO, M., MATSUZAKA, T., YATOH, S., KITAMINE, T., OKAZAKI, H., TAMURA, Y., SEKIYA, M., TAKAHASHI, A., HASTY, A.H., SATO, R., SONE, H., OSUGA, J., ISHIBASHI, S. & YAMADA, N. (2003). Cross-talk between peroxisome proliferator-activated receptor (PPAR) alpha and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. I. PPARs suppress sterol regulatory element binding protein-1c promoter through inhibition of LXR signaling. *Mol. Endocrinol.,* **17,** 1240–1254.

ZHOU, Y.T., GRAYBURN, P., KARIM, A., SHIMABUKURO, M., HIGA, M., BAETENS, D., ORCI, L. & UNGER, R.H. (2000). Lipotoxic heart disease in obese rats: implications for human obesity. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 1784–1789.

ZHOU, Y.T., SHIMABUKURO, M., WANG, M.Y., LEE, Y., HIGA, M., MILBURN, J.L., NEWGARD, C.B. & UNGER, R.H. (1998). Role of peroxisome proliferator-activated receptor alpha in disease of pancreatic beta cells. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 8898–8903.

(Received January 10, 2005
Revised February 18, 2005
Accepted April 4, 2005
Published online 9 May 2005)