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Journal of Nutritional Biochemistry 24 (2013) 419–427

Journal of Nutritional Biochemistry

Citrus unshiu peel extract ameliorates hyperglycemia and hepatic steatosis by altering inflammation and hepatic glucose- and lipid-regulating enzymes in *db*/*db* mice☆

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Received 23 September 2011; received in revised form 15 December 2011; accepted 22 December 2011

Abstract

Insulin resistance in Type 2 diabetes leads to hepatic steatosis that can accompanied by progressive inflammation of the liver. Citrus unshiu peel is a rich source of citrus flavonoids that possess anti-inflammatory, anti-diabetic and lipid-lowering effects. However, the ability of citrus unshiu peel ethanol extract (CPE) to improve hyperglycemia, adiposity and hepatic steatosis in Type 2 diabetes is unknown. Thus, we evaluated the effects of CPE on markers for glucose, lipid metabolism and inflammation in Type 2 diabetic mice. Male C57BL/KsJ-*db*/*db* mice were fed a normal diet with CPE (2 g/100 g diet) or rosiglitazone (0.001 g/100 g diet) for 6 weeks. Mice supplemented with the CPE showed a significant decrease in body weight gain, body fat mass and blood glucose level. The antihyperglycemic effect of CPE appeared to be partially mediated through the inhibition of hepatic gluconeogenic phosphoenolpyruvate carboxykinase mRNA expression and its activity and through the induction of insulin/glucagon secretion. CPE also ameliorated hepatic steatosis and hypertriglyceridemia via the inhibition of gene expression and activities of the lipogenic enzymes and the activation of fatty acid oxidation in the liver. These beneficial effects of CPE may be related to increased levels of anti-inflammatory adiponectin and interleukin (IL)-10, and decreased levels of pro-inflammatory markers (IL-6, monocyte chemotactic protein-1, interferon-γ and tumor necrosis factor-α) in the plasma or liver. Taken together, we suggest that CPE has the potential to improve both hyperglycemia and hepatic steatosis in Type 2 diabetes.

Keywords: Citrus unshiu peel extract; Type 2 diabetic *db*/*db* mice; Anti-diabetic; Lipid metabolism; Inflammation

1. Introduction

Diabetes is a global public health problem, and its prevalence is currently estimated to be about 6.4% worldwide [1]. In particular, there has been a dramatic increase in Type 2 diabetes in the past 2 decades [1]. Type 2 diabetes is characterized by hyperglycemia resulting from the combination of resistance to insulin action and inadequate insulin secretion. Insulin resistance is strongly associated with hepatic steatosis which is a relatively early condition of nonalcoholic fatty liver disease (NAFLD) and can lead to more severe NAFLD complications, such as steatohepatitis (steatosis plus inflammation and/or fibrosis) and cirrhosis [2]. Although it remains unclear whether hepatic steatosis is a consequence of insulin resistance or causes hepatic insulin resistance, patients with NAFLD are more prone to have insulin resistance and Type 2 diabetes [3], and the prevalence of the severe stage of NAFLD is high in Type 2 diabetes [4]. Most notably, insulin sensitizers such as thiazolidinediones and antioxidants improve Type 2 diabetes [5,6]. However, thiazolidinediones like rosiglitazone (RGZ) have some unfavorable complications including weight gain [7], hepatotoxicity [8] and congestive heart failure [9]. In recent years, medicinal plants that act as potent anti-inflammatory and antioxidant agent have attracted a lot of attention globally [10].

Citrus unshiu (*Citrus unshiu* Marcorv., Family: Rutaceae) is a seedless and easy-peeling Korean citrus fruit and it accounts for 30% of the total fruits produced in Korea. Its peels have been widely used as a folk medicine to improve bronchial and asthmatic conditions or blood circulation in Korea, China and Japan [11]. Previous studies reported that citrus unshiu peels have many pharmacologic activities including anti-inflammatory, antioxidant, anti-allergic and anti-cancer properties [12-15]. No studies have so far been done on its anti-diabetic and lipid-lowering effects. Citrus unshiu peel contains many phytochemicals such as hesperidin, naringin and nobiletin [16]. We previously demonstrated that hesperidin and niringin ameliorated hyperglycemia, dyslipidemia

☆ Funding Source: This work was supported by the Korea Ministry of Knowledge Economy and the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2011-0000920).
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http://dx.doi.org/10.1016/j.jnutbio.2011.12.009

and hepatic steatosis in Type 2 diabetic *db/db* mice by regulating the gene expression and activities of glucose- and lipid-regulating enzymes [17,18]. A recent study reported that nobiletin activated hepatic insulin sensitivity and attenuated dyslipidemia and atherosclerosis in low-density lipoprotein receptor knockout mice with diet-induced insulin resistance [19].

In light of these collective data, we hypothesized that citrus unshiu peel ethanol extract (CPE) would improve glucose homeostasis, hyperlipidemia and hepatic steatosis in *db/db* mice, which are a good model for Type 2 diabetes that displays many of the characteristics of the human disease, including hyperphagia, obesity, hyperglycemia, temporary hyperinsulinemia, hyperlipidemia and hepatic steatosis, due to leptin receptor mutations [20,21]. The effects of CPE were compared with those of RGZ, an oral anti-diabetic agent.

2. Materials and method

2.1. Preparation of extracts

Dried citrus unshiu peels were purchased from Omniherb (Yeongcheon, Korea). Samples for the study were prepared by adding 1 kg of 60% ethanol to 50 g of dried citrus peels: extraction was done at 70°C for 3 h, and then the solution was cooled and filtered with Whatman paper (No. 2). The filtered solution was concentrated at 60°C with a rotary vacuum evaporator (N-1000, Tokyo Rikakikai, Tokyo, Japan), and the concentrated sample was frozen and dried at −40°C with a freeze dryer (PVTFD10R, Ilshinbiobase, Yangju, Korea). The final weight of the lyophilized powder of the citrus peel ethanol extract (CPE) was 20.10 g with 40.2% yield. The major flavonoid was hesperidin (55.75 mg/g), and narirutin was the second most abundant flavonoid in CEP (21.86 mg/g). Other flavonoids including naringin were also found in CPE, although only in trace amounts.

2.2. Experimental animals

All animal procedures were in compliance and approved by the Ethic Committee for animal studies at Kyungpook National University. Thirty male C57BL/KsJ-*db/db* mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at 4 weeks of age. After acclimation period of 2 weeks, the mice were randomly divided into three groups (n=10) and fed the respective experimental diets for 6 weeks, as shown in Table 1: a normal control diet (CON) and a normal control diet with either rosiglitazone (RGZ; 0.001 g/100 g diet) or citrus unshiu peel ethanol extract (CPE; 2 g/100 g diet). The CPE was given as a supplement based on 5% dried powder of CPE/100 g diet.

All mice were maintained in a controlled light-dark cycle (12:12 hour) and constant temperature (24°C). They were given free access to food and distilled water and their food consumption and body weight were measured daily and weekly, respectively. After feeding with the experimental diets for 6 weeks, the mice were starved for 12 hours and anatomized under anesthesia by diethyl ether, and blood samples were taken from the inferior vena cava to determine blood and plasma biomarkers. The livers and adipose tissues were removed, washed, weighted and frozen at −70°C until analyzed.

2.3. Plasma parameters

The blood glucose concentration was monitored in the venous blood drawn from the tail vein using a glucometer (Gluco Dr supersensor, Allmedicus, Korea) after a 12-hour fast. The level of adiponectin (ELISA kit, Millipore, MA, USA) was determined with a quantitative sandwich enzyme immunoassay kit. The cytokines [interleukin (IL)-6, IL-10, monocyte chemotactic protein-1 (MCP-1), interferon (IFN)-γ, tumor necrosis factor (TNF)-α], insulin and glucagon were measured in the plasma, with a multiplex detection kit from Bio-Rad (Hercules, CA, USA). All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex, Austin, TX, USA) and data analyses were done with Bio-Plex Manager software version 4.1.1 (Bio-Rad, Hercules, CA, USA).

Table 1
Composition of experimental diets (%of diet)

	CON	CPE	RGZ
Casein	20	20	20
DL-Methionine	0.3	0.3	0.3
Sucrose	50	48.59	50
Cellulose powder	5	5	5
Cholinbitarate	0.2	0.2	0.2
AIN-76 mineral mixture	3.5	3.48	3.5
AIN-76 vitamin mixture	1	1	1
Corn starch	15	15	15
Corn oil	5	4.93	5
Citrus unshiu peel extract	-	2	-
Rosiglitazone	-	-	0.001
Total (%)	100	100	100

Yield of ethanol extract of citrus unshiu was 40.2%.

2.4. Plasma and hepatic lipids

The plasma cholesterol and triglyceride concentrations were determined with enzymatic kits (Sigma Diagnostics, Chemical, St. Louis, MO, USA). The free fatty acid (non-esterified fatty acid) was also measured with commercial assay kits (NEFA-Wako; Wako Pure Chemical Industries, Osaka, Japan).

The hepatic lipids were extracted [22], and then the dried lipid residues were dissolved in 1 ml of ethanol for triglyceride and cholesterol assays. Triton X-100 and a sodium cholate solution in distilled water were added to 200 μl of the dissolved lipid solution for emulsification. The hepatic triglyceride, cholesterol and free fatty acid contents were analyzed with the same enzymatic kit used for the plasma analysis.

2.5. Hepatic glucose-regulating enzyme activities and glycogen content

The glucokinase (GK) activity was determined from liver samples homogenized in 9 volumes of a buffer containing 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L KCl, 10 mmol/L mercaptoethanol and 1 mmol/L EDTA. Homogenates were centrifuged at 100,000×g for 1 h; the cytosol was used for the spectrophotometric assay as described by Davidson and Arion [23], whereby the formation of glucose-6-phosphate from glucose at 37°C was coupled to its oxidation by glucose-6-phosphate dehydrogenase (G6PD) and nicotinamide adenine dinucleotide (NAD). The glucose-6-phosphatase (G6Pase) activity was determined in the microsome with a spectrophotometric assay according to the method of Alegre et al. [24]. The reaction mixture contained the following: 100 mmol/L sodium Hepes (pH 6.5), 26.5 mmol/L glucose-6-phosphatate and 1.8 mmol/L EDTA, both previously adjusted to pH 6.5, 2 mmol/L $NADP^+$, 0.6 IU/L mutarotase, and 6 IU/L glucose dehydrogenase. The phosphoenolpyruvate carboxykinase (PEPCK) activity was determined according to a method described by Bentle and Lardy [25]. The reaction mixture contained the following in a 1 ml final volume: 77 mol/L sodium HEPES, 1 mmol/L IDP, 1 mmol/L $MnCl_2$, 1 mmol/L dithiothreitol, 0.25 mmol/L NADH, 2 mmol/L phosphoenolpyruvate, 50 mmol/L $NaHCO_3$ and 7.2 U of malic dehydrogenase. The glycogen level was determined as previously described by Seifter et al. [26]. Briefly, liver tissue was homogenized in 5 volumes of an ice-cold KOH solution [30% (w/v)] and dissolved in a boiling water-bath (100°C) for 30 min. The glycogen was precipitated with ethanol, and then pelleted, washed and resolubilized in distilled water. The glycogen level was measured at 620 nm after treatment with an anthrone reagent [2 g anthrone/1 L of 95% (v/v) H_2SO_4].

2.6. Hepatic lipid-regulating enzyme activities

Fatty acid β-oxidation activity (β-oxidation) was measured spectrophotometrically by monitoring the reduction of NAD to NADH in the presence of palmitoyl-CoA as described by Lazarow [27]. The carnitine palmitoyltransferase (CPT) activity was determined according to the method by Markwell et al. [28]. The results were expressed as nmol/min per mg of protein. Fatty acid synthase (FAS) activity was determined according to the method described by Carl et al [29] by monitoring the malonyl-CoA-dependent oxidation of NADPH at 340 nm, where the activity represents the oxidized NADPH nmol/min per mg of protein. The malic enzyme (ME) activity was determined as previously described by Ochoa [30]. The cytosolic enzyme was mixed with 0.2 mM triethanolamine buffer (pH 7.4), 1.5 mM L-malate, 12 mM $MnCl_2$ and 680 μM $NDAP^+$, which was then measured for 1 min at 340 nm (26°C) on a spectrophotometer. The G6PD activity was assayed by spectrophotometric methods according to the procedures described by Pitkänen et al. [31], where the activity was expressed as the reduced NADPH nmol/min per mg of protein. The phosphatidate phosphohydrolase (PAP) activity was determined using the method of Walton and Possmayer [32].

2.7. RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

The liver was homogenized in TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) and total RNA was isolated according to the manufacturer's instructions. DNase digestion was used to remove any DNA contamination and the RNA was re-precipitated in ethanol to ensure no phenol contamination. For quality control, RNA purity and integrity were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Equal amounts of the RNA from each experimental group were pooled to normalize individual differences.

Total RNA (1 μg) was reverse-transcribed into cDNA using the QuantiTect® reverse transcription kit (Qiagen, Germany). mRNA expression was quantified by real-time quantitative PCR, using the SYBR green PCR kit (Qiagen, Germany) and the CFX96TM

real-time system (Bio-Rad, Hercules, CA, USA). The following gene-specific primers were used: for CPT, 5′-ATC TGG ATG GCT ATG GTC AAG GTC-3′ (forward), 5′-GTG CTG TCA TGC GTT GGA AGT C-3′ (reverse); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACA ATG AAT ACG GCT ACA GCA ACA G-3′ (forward), 5′-GGT GGT CCA GGG TTT CTT ACT CC-3′ (reverse); for FAS, 5′-CGC TCC TCG CTT GTC GTC TG -3′ (forward), 5′-AGC CTT CCA TCT CCT GTC ATC ATC-3′ (reverse); for GK, 5′-CAG GAC AGT GGA GCG TGA AGA C-3′ (forward), 5′-TTA CAG GGA AGG AGA AGG TGA AGC-3′ (reverse); for G6Pase, 5′-GGA GGA AGG ATG GAG GAA GGA ATG-3′ (forward), 5′-GGT CAG CAA TCA CAG ACA CAA GG-3′ (reverse); for G6PD, 5′-AAC GCC TTC TAT GTC CTC TTT C-3′ (forward), 5′-GTG GTT GCT GTA GTA GTC GGT GTC C-3′ (reverse); for 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), 5′-TTC ACG CTC ATA GTC GCT GGA TAG-3′ (forward), 5′-TGG TTC AAT TCT CTT GGA CAC ATC TTC-3′ (reverse); for MCP-1, 5′-TTC CTC CAC CAC CAT GCA G-3′ (forward), 5′-CCA GCC GGC AAC TGT GA-3′ (reverse); for ME, 5′-AGG GCA CAT TGC TTC AGT TC-3′ (forward), 5′-TGT ACA GGG CCA GTT TAC CC-3′ (reverse); for PEPCK, 5′-TGC CTC TCT CCA CAC CAT TGC-3′ (forward), 5′-TGC CTT CCA CGA ACT TCC TCA C-3′ (reverse). Cycle thresholds were determined based on the SYBR green emission intensity during the exponential phase. Using the $2^{-\Delta\Delta Ct}$ method, the fold changes were calculated; transcripts of GAPDH were also amplified from the samples in order to assure normalized real-time quantitative RT-PCR detection [33].

2.8. Morphological analysis of liver and epididymal fat

Liver and epididymal fat were removed and fixed in a buffer solution of 10% formalin. Fixed tissues were processed routinely for paraffin embedding, and 4-μm sections were prepared and dyed with hematoxylin-eosin. Stained areas were viewed using an optical microscope with a magnifying power of ×200.

2.9. Statistical analysis

All data were presented as the mean±S.E. The data were evaluated by a one-way analysis of variance SPSS program and by determining the differences between the means with Duncan's multiple-range test. Values were considered statistically significant at $P<.05$.

3. Results

3.1. Food intake, body weight gain and body fat mass

Supplementation with CPE suppressed body weight gain over 6 weeks in Type 2 diabetic *db*/*db* mice, whereas RGZ markedly increased the body weight gain compared to both the control and CPE-supplemented *db*/*db* mice (Fig. 1A and B). The average daily food intake throughout the experimental period was not significantly altered by CPE feeding (CON: 2.90±0.13 g, CPE: 3.19±0.16 g); however, RGZ significantly lowered the daily food intake (RGZ: 2.00 ±0.13 g). Thus, mice fed the CPE showed a significant lower food efficiency ratio (FER) compared to the control group, but RGZ significantly increased it by 2.9-fold, suggesting greater weight gain efficiency (Fig. 1C). Furthermore, supplementation with CPE effectively reduced total white adipose tissue (WAT) weight and epididymal WAT size in the *db*/*db* mice. In contrast, RGZ-supplemented mice had a significant increase in total WAT weight and epididymal WAT size compared to the control and CPE-supplemented mice (Fig. 1D and 1E).

3.2. Blood glucose and plasma hormones

All *db*/*db* mice were diabetic when the experiment began, as indicated by the fasting blood glucose level (≥12 mmol/L). After 6 weeks of experimental feeding, the mice that were fed CPE or RGZ showed a significant reduction in the fasting blood glucose level compared to the control mice (Table 2). The hepatic glycogen content was also significantly lower in the CPE- or RGZ-supplemented *db*/*db* mice than in the control *db*/*db* mice (Table 2). Supplementation with

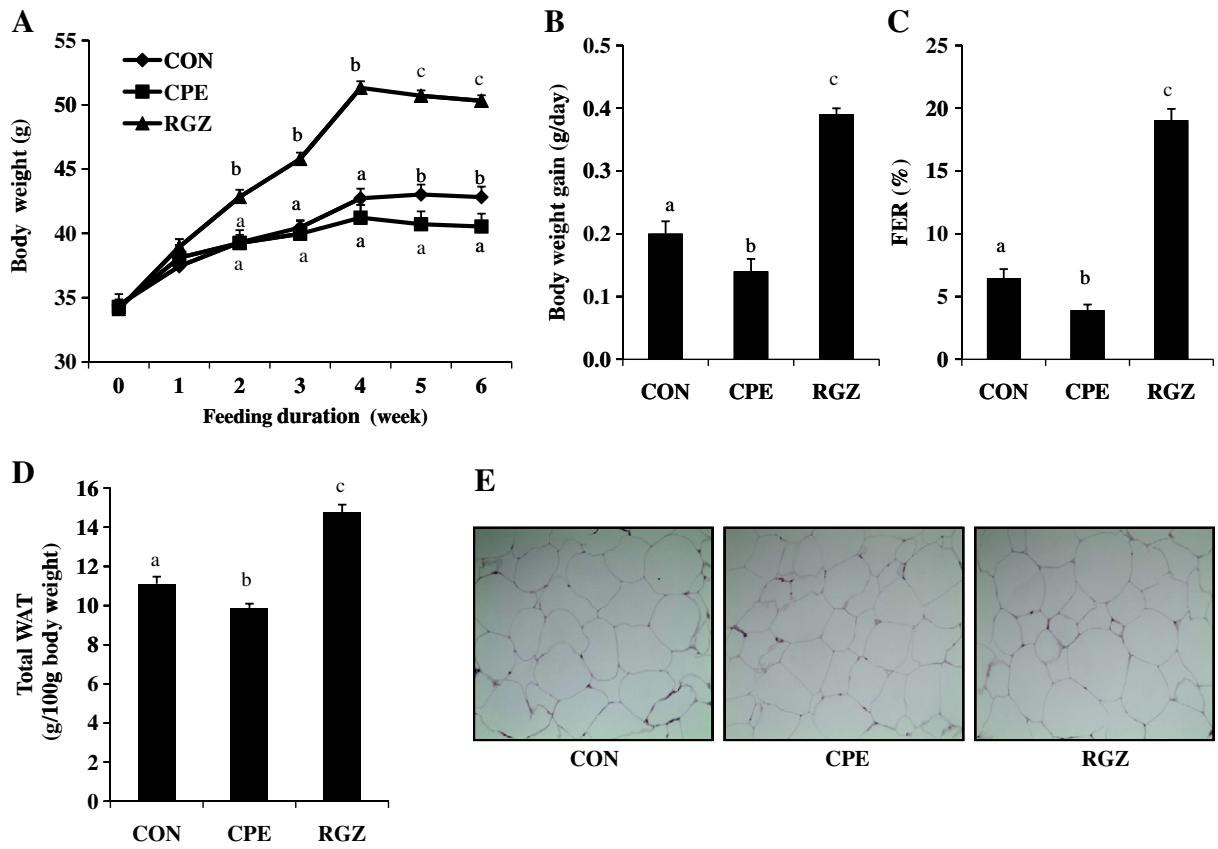

Fig. 1. Effect of dietary CPE on body weight change (A), body weight gain (B), food efficiency (C), Total WAT weight (D) and epididymal WAT morphology (E) in *db*/*db* mice. C57BL6/KsJ-*db*/*db* mice were fed a normal diet with or without CPE (2 g/100 g diet) and RGZ (0.001 g/100 g diet) for 6 weeks ($n=10$ for each group). Original magnification ×100. Data are Mean±S.E. [abc]Means not sharing a common letter are significantly different groups at $P<.05$.

Table 2
Effect of dietary CPE on blood glucose, hepatic glycogen and plasma hormone and lipid levels in *db*/*db* mice

	CON	CPE	RGZ
Blood glucose (mmol/L)			
Initial	12.17±1.24	11.60±1.35	12.22±1.02
Final	30.58±0.78[a]	26.00±2.08[b]	17.82±0.73[c]
Hepatic glycogen (mg/g liver)	31.74±1.65[a]	23.20±2.53[b]	10.00±0.77[c]
Plasma insulin (ng/ml)	4.86±0.59[a]	7.81±1.21[ab]	18.38±2.46[b]
Plasma glucagon	0.36±0.03[ab]	0.28±0.06[a]	0.43±0.04[b]
Insulin/glucagon	13.50±0.97[a]	27.89±4.56[b]	42.74±2.55[c]
Plasma lipids (mmol/l)			
Free fatty acid	0.65±0.06[a]	0.55±0.07[a]	0.36±0.05[b]
Triglyceride	3.15±0.39[a]	2.00±0.40[b]	1.57±0.18[b]
Total cholesterol	4.63±0.22[ab]	4.13±0.20[a]	5.32±0.41[b]

Mean±SE (n=10 for each group). [abc]Means in the row not sharing a common letter are significantly different groups at P<.05.

CPE and RGZ significantly increased the plasma insulin level compared to the control group by 1.6- and 3.8-fold, respectively (Table 2). In contrast to the plasma insulin level, the mice that were fed the CPE had a 22% decrease in the plasma glucagon level compared to the control mice and, thus, led to an increased insulin/glucagon ratio (Table 2). The RGZ also resulted in a significant increase in insulin/glucagon ratio, although it tended to increase the plasma glucagon level by 19% compared to the control mice (Table 2).

3.3. Plasma and hepatic lipids levels

At the end of the study, supplementation with CPE or RGZ significantly lowered the plasma triglyceride level in *db*/*db* mice (Table 2). The plasma free fatty acid level was also significantly lowered in the RGZ-fed mice, whereas no change was observed in CPE-fed mice (Table 2). Both CPE and RGZ did not affect the plasma total cholesterol level (Table 2).

In contrast to the plasma triglyceride and free fatty acid levels, supplementation with RGZ significantly increased these levels in the liver of *db*/*db* mice (Fig. 2A and B). However, dietary CPE led to a significant decrease in hepatic triglyceride, cholesterol and free fatty acid contents compared to the control and RGZ group (Fig. 2A–C). Morphological analysis of the liver also indicated lipid droplet accumulation was most pronounced in the RGZ-fed mice, while supplementation with CPE resulted in a reduced number of lipid droplets and a reduced liver size compared to the control and RGZ-fed *db*/*db* mice (Fig. 2E). Furthermore, livers from the mice that were fed the RGZ weighed more than those in the control group, whereas the CPE diet led to a significant decrease in the liver weight (Fig. 2D).

3.4. Hepatic glucose-regulating enzyme activities and mRNA expression

To further examine the mechanism through which CPE ameliorates hyperglycemia in *db*/*db* mice, we examined the mRNA expression and activities of enzymes that can regulate glucose homeostasis in the liver (Fig. 3A and B). RGZ significantly decreased gluconeogenic G6Pase activity in the liver, whereas the hepatic GK activity in the RGZ group was higher compared to the control group. Supplementation with dietary CPE did not significantly alter hepatic G6Pase and GK activities, yet it led to a significant decrease in the activity of hepatic PEPCK, another gluconeogenic enzyme. The changes in the mRNA levels of glucose metabolic enzymes were similar to the respective enzyme activities in the liver.

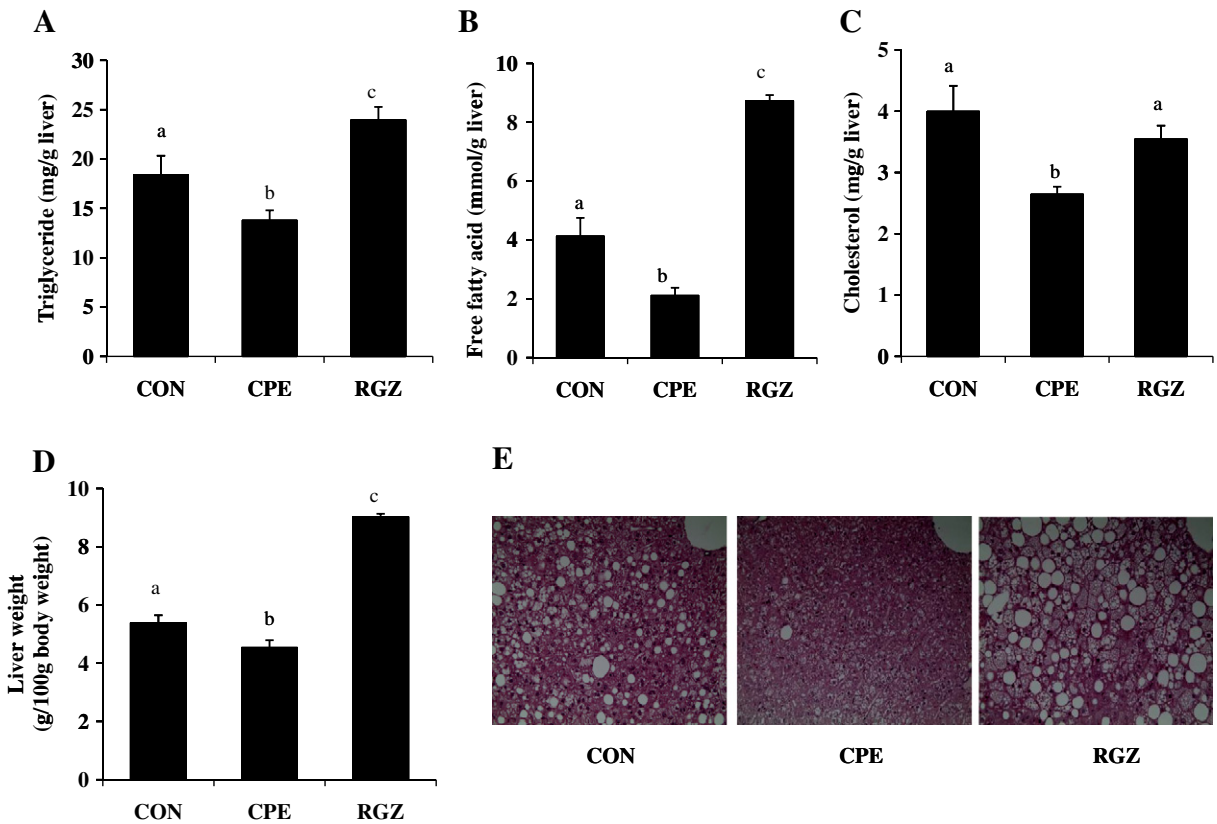

Fig. 2. Effect of dietary CPE on hepatic lipid content (A–C), liver weight (D) and hepatic morphology (E) in C57BL6/KsJ-*db*/*db* mice. Hepatic lipid droplet accumulation in fixed tranverse liver sections stained with hematoxylin and eosin. Original magnification ×200. Data are Mean±S.E. (n=10 for each group). [abc]Means not sharing a common letter are significantly different groups at P<.05.

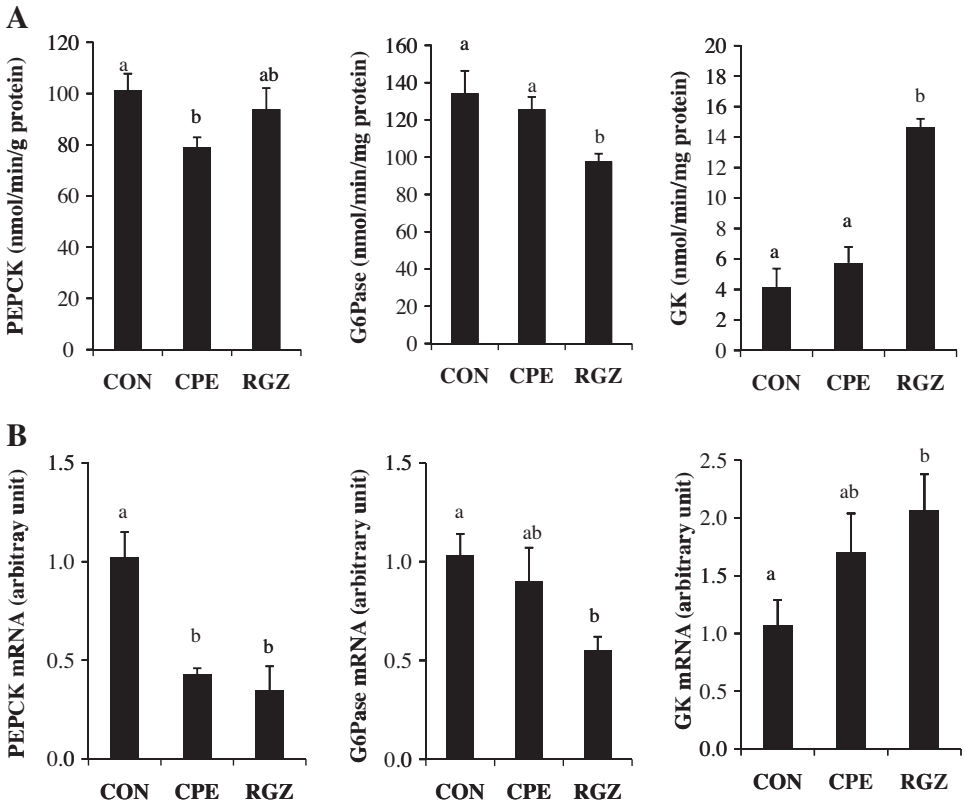

Fig. 3. Effect of dietary CPE on hepatic glucose-regulating enzyme activity (A) and gene expression (B) in *db*/*db* mice. Data are Mean±S.E. (n=10 for each group). [ab]Means not sharing a common letter are significantly different groups at P<.05.

3.5. Hepatic lipid regulating enzyme activities and mRNA expression

Next, we determined the gene expression and activities of enzymes regulating lipogenesis and fatty acid oxidation in the liver to investigate how CPE decreases hepatic fat accumulation (Table 3 and Fig. 4). The activities of enzymes for *de novo* fatty acid synthesis including FAS, ME and G6PD were markedly higher in the RGZ-supplemented *db*/*db* mice, whereas the mice that were fed the CPE led to a significant decrease in the hepatic FAS and ME activities and their respective gene expression compared to the control or RGZ groups. Furthermore, activity of PAP, a key enzyme in triglyceride synthesis, and mRNA level of HMGR, a rate-limiting enzyme in cholesterol synthesis, were significantly lowered in the CPE group. Hepatic CPT mRNA expression along with fatty acid β-oxidation was also significantly elevated in the CPE- or RGZ-supplemented group than in the control group (Fig. 4). Accordingly, these results suggest that CPE decreased hepatic lipid accumulation by lowering lipogenesis and activating fatty acid oxidation in the liver of *db*/*db* mice.

Table 3
Effect of dietary CPE on hepatic lipid-regulating enzyme activity in *db*/*db* mice

	CON	CPE	RGZ
Fatty acid synthesis (nmol/min per mg of protein)			
FAS	14.92±0.77[a]	12.15±0.80[b]	18.85±0.96[c]
ME	22.97±1.56[a]	17.74±0.67[b]	45.94±1.95[c]
G6PD	5.16±0.38[a]	5.80±0.43[a]	10.88±0.30[b]
Triglyceride synthesis (nmol/min per mg of protein)			
PAP	12.52±0.32[a]	10.96±0.47[b]	13.32±0.24[a]
Fatty acid oxidation (nmol/min per mg of protein)			
β-oxidation	14.83±0.85[a]	21.82±1.23[b]	31.28±2.24[c]
CPT	14.83±1.61	18.24±1.01	15.05±0.81

Mean±SE (n=10 for each group). [abc]Means in the row not sharing a common letter are significantly different groups at P<.05.

3.6. Blood and hepatic inflammatory biomarkers

Type 2 diabetes and hepatic steatosis is associated with inflammation. To explore the effect of the CPE on inflammation, well-defined pro-and anti-inflammatory markers in the plasma and liver were compared (Fig. 5). The levels of the pro-inflammatory markers such as IL-6, TNF-α and IFN-γ were significantly lower in the plasma of the CPE and RGZ groups compared to the control group (Fig. 5B). CPE-supplemented mice also showed a significant decrease in the plasma MCP-1 level and hepatic MCP-1 mRNA expression, but RGZ did not alter this pro-inflammatory marker in plasma and liver (Fig. 5B and C). Moreover, the plasma anti-inflammatory adiponectin and IL-10 levels increased 79% and 65%, respectively, after supplementation with CPE in *db*/*db* mice; however, RGZ feeding lowered the plasma adiponectin level by 30% compared to the control group (Fig. 5A). These findings indicate that dietary CPE attenuated diabetes-induced inflammatory responses.

4. Discussion

This study was designed to investigate the effects of CPE on hyperglycemia, dyslipidemia and hepatic steatosis in Type 2 diabetic *db*/*db* mice. We have reported that hesperidin, the main flavonoid found in CPE, has important roles in preventing the progression of hyperglycemia, partly by increasing glycolysis and inhibiting gluconeogenesis in the liver [17]. Other recent studies have also shown that hesperidin improves glucose metabolism by altering the activities of hepatic glucose-regulating enzymes in rat

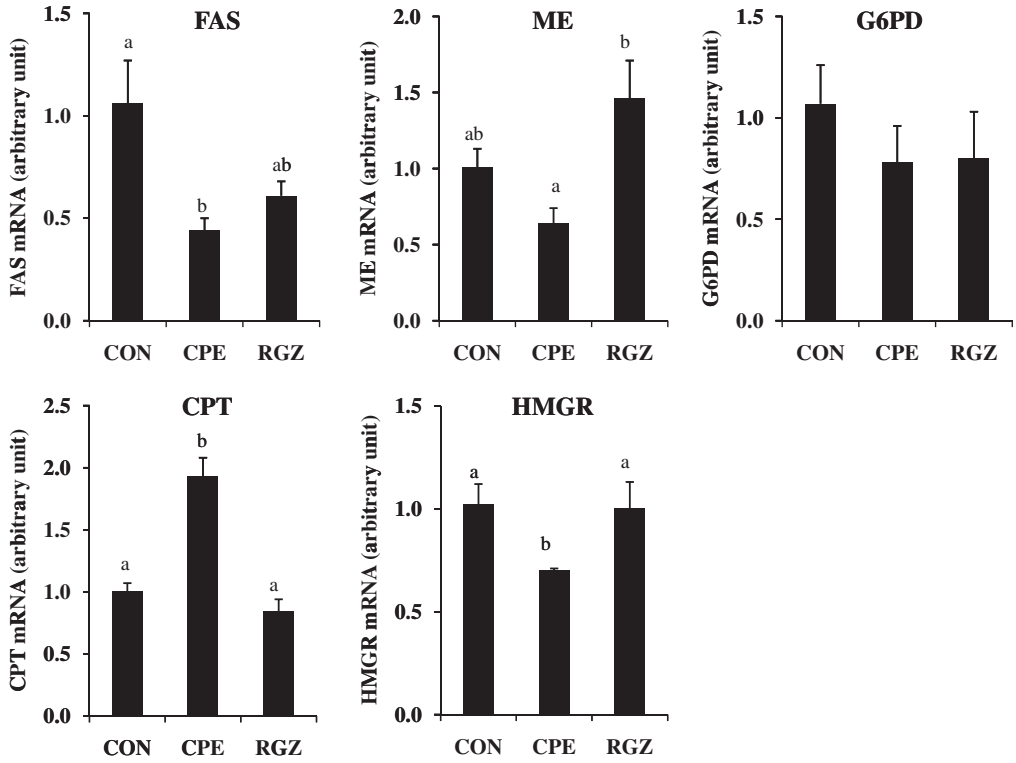

Fig. 4 Effect of dietary CPE on hepatic lipid-regulating gene expression in *db*/*db* mice. Data are Mean±S.E. (n=10 for each group). [ab]Means not sharing a common letter are significantly different groups at P<.05.

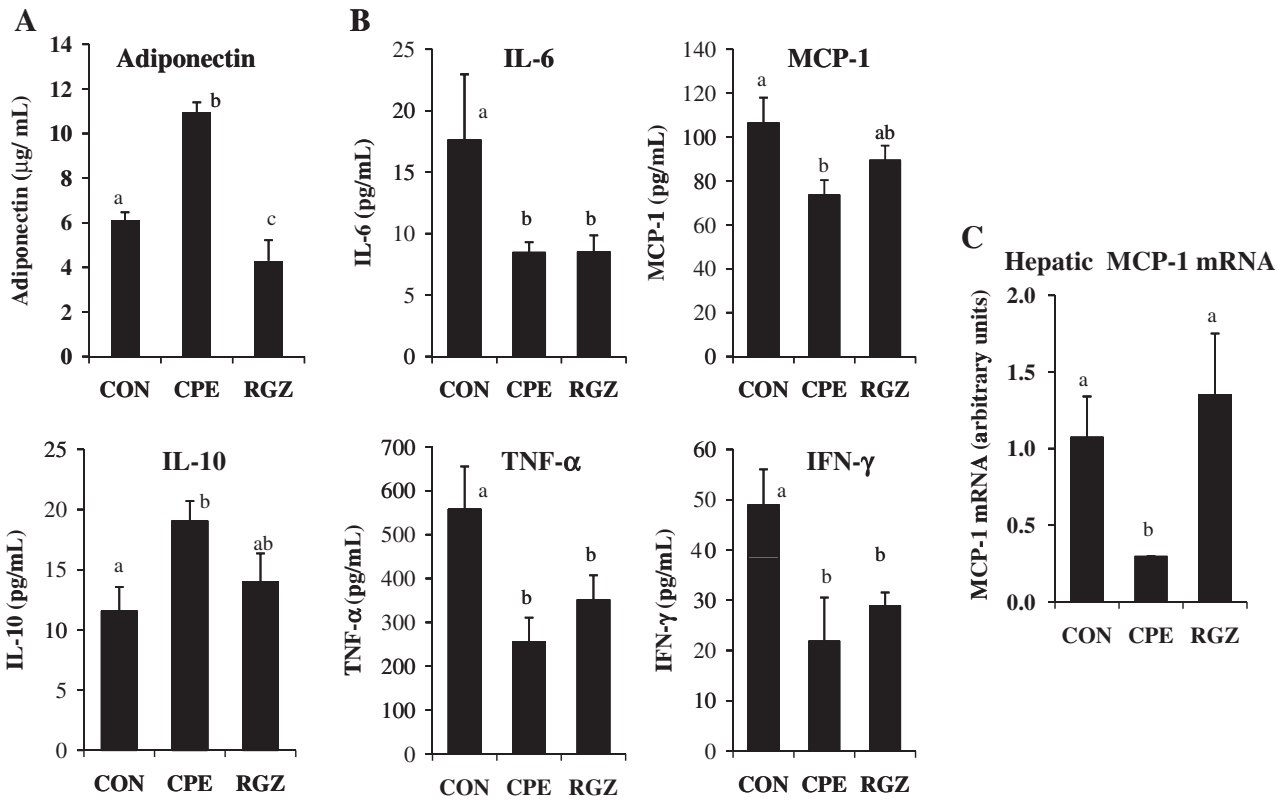

Fig. 5. Effect of dietary CPE on inflammatory markers in plasma (A, B) and liver (C) of *db*/*db* mice. (A) Concentration of plasma anti-inflammatory markers, adiponectin and IL-10. (B) Concentration of pro-inflammatory IL-6, MCP-1, TNF-α and IFN-γ in plasma. (C) Hepatic MCP-1 mRNA expression. Data are Mean±SE (n=10 for each group). [abc]Means not sharing a common letter are significantly different groups at P<.05.

models with type 1 [34] and Type 2 diabetes [35]. Moreover, peel extract of citrus sinensis (sweet orange), a citrus fruit containing hesperidin, ameliorated blood glucose in human [36] and animals [37,38]. In the present study, the supplementation with 2% CPE for 6 weeks significantly lowered the fasting blood glucose level compared to the control *db*/*db* mice. The hypoglycemic action of the CPE is likely associated with a marked decrease in the PEPCK activity and its gene expression in the liver. PEPCK is a key enzyme involved in hepatic gluconeogenesis and the increased hepatic glucose production is a major cause of fasting hyperglycemia in Type 2 diabetes [39]. Overexpression of the hepatic PEPCK gene in mice induced insulin resistance and a diabetic phenotype [40]. A high PEPCK gene expression and its activity were observed in the liver of *db*/*db* mice [41]. The PEPCK activity is primarily regulated by transcription of the PEPCK gene and by several hormones modulating hepatic PEPCK at the transcriptional level [42]. Glucagon activates hepatic PEPCK gene transcription, whereas insulin inhibits glucagon action [42]. Kodama et al. [43] reported that the plasma glucagon/insulin ratio but not the absolute concentration of either hormone paralleled the blood glucose level and hepatic gluconeogenic enzyme activity. Taken together, these data suggest that the increased plasma insulin/glucagon ratio in the CPE-supplemented *db*/*db* mice may be related to the suppression of hepatic PEPCK activity and contribute to the anti-hyperglycemia.

The enhanced gluconeogenesis is related to the net accumulation of hepatic glycogen in diabetes [44], and a liver-specific inhibition of PEPCK in mice dramatically decreased the hepatic glycogen content [45,46]. An excessive glycogen accumulation in the liver is observed in diabetic patients [47], and *db*/*db* mice [48]. In the current study, dietary CPE significantly lowered hepatic glycogen content. Thus, the decrease in hepatic glycogen content in CPE-supplemented *db*/*db* mice may reflect a diminished glycogen synthesis from gluconeogenic precursors through the suppression of PEPCK activity, although we did not test the glycogen synthesis pathway. Here, we also found that *db*/*db* mice supplemented with CPE exhibited a significant lower liver weight/body weight compared to the control and RGZ groups. The excessive glycogen content in the liver causes hepatomegaly in diabetic patients [49]. The hepatic lipid accumulation is also the other major cause of hepatomegaly in diabetic patients [50] and *db*/*db* mice [51].

Hepatic steatosis, an excessive accumulation of lipid in the liver, is often associated with resistance to the actions of insulin on hepatic gluconeogenesis [52]. In Type 2 diabetes, insulin fails to suppress gluconeogenesis but continues to activate de novo lipogenesis, producing the combination of hyperglycemia, hepatic steatosis and hypertriglyceridemia [52]. Additionally, hepatic fatty acid oxidation was decreased in insulin resistant [53], and activation of fatty acid oxidation improved insulin sensitivity and reduced hepatic lipid accumulation in obese mice [54]. Accordingly, in conditions of insulin resistance and Type 2 diabetes, hepatic steatosis can occur because of abnormally enhanced de novo lipogenesis as well as decreased fatty acid oxidation [55]. In the current study, CPE ameliorated hepatic steatosis, as determined by dramatically decreased the hepatic fatty acid, triglyceride and cholesterol contents and a decrease in the lipid droplet size and number. These results were seemingly accompanied by the decreased activities of lipogenic enzymes, including FAS, ME, and PAP, as well as enhanced hepatic fatty acid oxidation. Furthermore, dietary CPE significantly down-regulated expression of genes involved in the synthesis of fatty acid (FAS, ME) and cholesterol (HMGR), whereas mRNA expression of CPT, a key regulatory enzyme for fatty acid oxidation, was up-regulated by CPE feeding. Accordingly, the coordinated action of improving hepatic lipogenesis and fatty acid oxidation seemed to contribute to the beneficial effects of CPE on hepatic steatosis in *db*/*db* mice, which resulted in reduced plasma triglyceride level, since the increased triglyceride synthesis in the liver promotes the secretion of triglyceride-enriched very low-density lipoprotein [56]. These findings are supported by a previous study [57] which demonstrated citrus unshiu segment membrane (0.5%, w/w) feeding for 7 weeks improved hepatic steatosis and hypertriglyceridemia in *db*/*db* mice. Moreover, hesperidin decreased plasma and hepatic triglyceride levels in Type 2 diabetic rats partly by activating the gene expression of peroxisome proliferators-activated receptor α which activates hepatic fatty acid oxidation [35].

Chronic inflammation is causally linked to insulin resistance and Type 2 diabetes [58]. Elevated levels of plasma pro-inflammatory cytokines (TNFα, IL-6, IFN-γ) and a chemokine (MCP-1) were observed in patient and animals with Type 2 diabetes [51,59-61]. In particular, MCP-1 [60] has an integral role in the pathogenesis of insulin resistant-related diseases including NAFLD. In contrast, circulating IL-10 was decreased in metabolic syndrome [62], and a low IL-10 production was associated with hyperglycemia in Type 2 diabetes [63]. Our data indicates that dietary CPE seems to have a favorable effect on the inflammatory status in *db*/*db* mice, since the plasma TNF-α, IL-6, MCP-1 and IFN-γ levels as well as hepatic MCP-1 mRNA level were decreased but the plasma IL-10 level was increased.

Some inflammatory cytokines such as TNF-α and IL-6 inhibit production of adiponectin [64]. Adiponectin itself has also anti-inflammatory effects through inhibition of TNF-α, IFN-γ and IL-6 as well as induction of IL-10 [65,66]. In addition, adiponectin enhances insulin action [64] and has a protective role in hepatic steatosis, since it enhances fatty acid oxidation and inhibits hepatic fatty acid uptake [67]. Circulating adiponectin was lower in patients with hepatic steatosis [68], and recombinant adiponectin improved hepatic steatosis and hepatomegaly in *ob*/*ob* mice. [69]. *db*/*db* mice also exhibited a lower serum adiponectin level compared to C57BL/6J mice [51], and in this study, we observed that CPE-supplemented *db*/*db* mice showed increased plasma adiponectin level compared to the control *db*/*db* mice. Together these findings suggest that the decreased pro-inflammatory markers and increased anti-inflammatory markers by dietary CPE partially contributes to the protection of hepatic steatosis and improvement in insulin resistance in *db*/*db* mice. Similarly, hesperidin significantly increased serum adiponectin level in Type 2 diabetic rats [35], and nobiletin increased adipose adiponectin mRNA expression in *ob*/*ob* mice [70]. However, it is difficult to assess whether the changes in plasma inflammatory markers are direct effects of less adiposity and hypertrophy by CPE, because pro-inflammatory adipokines such as IL-6, TNF-α and MCP-1 were positively correlated with adipocyte size, whereas there was a decrease in IL-10 secretion with increasing cell size [71].

Our data shows that the RGZ appeared to have a more potent hypoglycemic effect than dietary CPE in *db*/*db* mice. However, RGZ led to hepatomegaly and hepatic steatosis in accordance with a previous study [72]. The RGZ-induced hepatic steatosis was accompanied by activating activities of lipogenic enzymes, including FAS, ME, and G6PD, although it also activated hepatic fatty acid oxidation. Furthermore, as expected, RGZ-supplemented mice showed hyperphagia, adiposity, and overweight.

We conclude that dietary CPE can ameliorate hyperglycemia through its ability to enhance insulin/glucagon secretion and to decrease hepatic PEPCK gene expression and its activity in *db*/*db* mice. Furthermore, CPE improves hypertriglyceridemia and hepatic steatosis through inhibition of de novo lipogenic enzyme activity and its mRNA expression as well as activation of fatty acid oxidation in the liver. It seems likely that CPE's anti-inflammatory effects may be helpful in preventing or delaying the development of Type 2 diabetes and its complications such as hepatic steatosis.

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