Modulating Effects of Chlorogenic Acid on Lipids and Glucose Metabolism and Expression of Hepatic Peroxisome Proliferator-activated Receptor-α in Golden Hamsters Fed on High Fat Diet[1]

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Objective To examine the effects of chlorogenic acid (CGA) on lipid and glucose metabolism under a high dietary fat burden and to explore the possible role of peroxisome proliferator-activated receptor-α (PPAR-α) in these effects. **Methods** Twenty male golden hamsters were randomly divided into CGA treatment group (n=10, given peritoneal injection of CGA solution prepared with PBS, 80 mg CGA/kg body weight daily), and control group (n=10, given PBS i.p. at the average volume of the treatment group). Animals in both groups were given 15% high fat diet. Eight weeks after treatment with CGA, the level of biochemical parameters in fasting serum and tissues and the expression of hepatic mRNA and protein PPAR-α were determined. **Results** Eight weeks after treatment with CGA, the levels of fasting serum triglyceride (TG), free fatty acid (FFA), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), glucose (FSG), and insulin (FSI) were significantly lower in the GGA treatment group than in the control group. CGA also led to higher activity of hepatic lipase (HL), lower contents of TG and FFA in liver, and lower activity of lipoprotein lipase (LPL) in skeletal muscle. Furthermore, CGA significantly elevated significantly elevated the expression level of mRNA and protein expression in hepatic PPAR-α. **Conclusion** CGA can modify lipids and glucose metabolism, which may be attributed to PPAR-α facilitated lipid clearance in liver and improved insulin sensitivity.

Key words: Chlorogenic acid; Golden hamster; High fat diet; Hypolipidemic effect; Hypoglycemic effect; Lipids clearance; FFA drainage; PPAR-α; Insulin sensitivity

INTRODUCTION

High dietary fat load induces dyslipidemia and glycometabolic disorders. When flux of fat (as triglyceride and triglyceride-derived free fatty acid) in circulation is above the rate of its utilization and oxidation, its excessive components will go elsewhere and deposit in other non-adipose and glucose-metabolizing tissues such as liver, skeletal muscle, pancreatic beta-cells, and eventually lead to impairment of insulin sensitivity[1-2]. Therefore, dyslipidemia can induce glycometabolic disorders[3], and agents such as drugs or phytochemical substances that promote clearance of blood triglyceride (TG) or free fatty acid (FFA) can modulate dyslipidemia and glycometabolic disorders.

Chlorogenic acid (CGA) is a kind of phenolic acid widespread in plant food and coffee drinks[4]. Other than reducing risk of cardiovascular diseases through its strong antioxidative property[5-8], CGA has been recently claimed to modulate lipid and glucose metabolism *in vivo* in both healthy and genetically metabolic disordered conditions[9-11]. It has been found that when CGA is administrated either intravenously or orally, it could be picked up by the liver, and might exert its modulating effects by improving hepatic metabolic functions, such as increasing insulin sensibility or activating hepatic TG metabolism[10-12]. However, the specific underlying mechanism remains unclear. It may be related to some upstream regulators on the common metabolic pathway of lipids and glucose. Peroxisome roliferator-activated receptor (PPAR) is one of these key regulators. Once activated by lipoid substance,

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they can regulate the expression of genes that control lipid and glucose metabolism homeostasis[13]. There are three proteins in the PPAR subfamily: PPARα, β/δ, and γ. PPAR-α is a kind of nuclear receptor that regulates binding, transport, oxidation and synthesis of FFA[14]. After activated, PPAR-α can increase the activity of FFA oxidation enzymes, elevate fat energy utilization in the liver and muscles, increase insulin sensitivity and decrease insulin resistance[15]. It was reported that CGA has inhibitory activity on enzymes involved in FFA synthesis *in vitro*[16]. Therefore, we hypothesize that CGA might modulate lipid and glucose metabolism through PPAR-α mediated lipid-lowering and subsequent insulin sensitizing effect. Since hamsters react to dietary lipids in a fashion similar to humans, and are successfully used in studies of diabetic dyslipidemia[17], we used golden hamsters as an appropriate model to examine the modulating effects of CGA on lipid and glucose metabolism under a high dietary fat burden and to explore the possible role of PPAR-α in these effects.

MATERIALS AND METHODS

Animals and Experimental Treatment

Twenty male golden hamsters weighing 60-80 g, purchased from Beijing Vitalriver Experimental Animal Technique Ltd (China), were housed in plastic-bottomed cages with reticulate stainless steel covers at 22±1 ℃ and 40%-60% relative humidity in a 12 h light/dark cycle. One week after acclimatizing feeding, the animals were randomly divided into CGA treatment group (n=10) and control group (n=10). Animals in the CGA treatment group were given CGA (Acros Ltd., USA) injection i.p. at a dose of 80 mg/kg body weight daily.

Cohort studies showed that habitual coffee consumption was inversely related with type 2 diabetes[18-20], and CGA intake could reach 1000 mg/day in habitual coffee drinkers[21], corresponding to about 14 mg/kg body weight daily for a man of 70 kg body weight. The dose for hamsters is generally 5-7 times that for humans, about 70-100 mg/kg body weight daily CGA for hamsters can be calculated. In this study, 80 mg/kg body weight daily was chosen.

A CGA solution (10 mg/mL, pH 7.2-7.4) was prepared with phosphate buffered saline (PBS). Animals in the control group were given PBS (i.p.) at the average volume for the CGA treatment group. From the first day of treatment, animals in both groups were given 15% high fat diet. One kilogram of the diet was made by mixing 900 g commercial normal diet powder (Experimental Animal Center, Chinese Academy of Preventive Medicine) with 100 g lard (obtained from local supermarket). The final composition of the diet is given in Table 1. Animal body weight and food intake were recorded daily.

This study was approved by the Animal Care and Use Committee of Peking University. All experimental protocols were in compliance with "The Guide for the Care and Use of Laboratory Animals" (National Academy Press, NIH Publication No. 85-23, 1996).

TABLE 1

Composition of the High Fat Diet

Ingredients	Content (g/kg)
Casein (Vitamin Free)	164
Soybean Oil	50
Lard	100
Sucrose	100
Corn Starch	348
Ash Content	70
Cellulose	40
Water	80
L-Cystine	3
Choline	1
Mineral Mixture (AIN-93G)	34
Vitamin Mixture (AIN-93G)	10

Note. Fat energy ratio 35.54%; carbohydrate energy ratio 47.18%; protein energy ratio 17.28%.

Sample Collection

Eight weeks after treatment with CGA, each animal was fasted for 12 h before anesthetized with 10% chloral hydrate (3.5 mL/kg, i.p.), and killed by exsanguination from femoral artery. Blood was centrifuged at 2000 rpm for 10 min at 4 ℃ and serum was collected. After blood collection, liver, visceral adipose tissue around the kidney, testicle and gastrocnemius muscle were removed and stored at -80 ℃ for later determination of tissue lipid content, tissue lipase activity and PPAR-α expression. Adipose tissue was weighed before storage.

Measurement of Serum Lipid Level

Serum TG level was measured by GPO-PAP enzymatic colorimetric analysis, total cholesterol (TC) level by CHOD-PAP enzymatic colorimetric analysis, low density lipoprotein-cholesterol (LDL-C) level with the PVS precipitation method, and high density

lipoprotein-cholesterol (HDL-C) level with the PTA-Mg2+ precipitation method[22] using corresponding kits (Biosino Biotechnology Company Ltd., Beijing, China). FFA content was determined with the ferment method using FFA kit (Kyowa Medex, Japan).

Measurement of Fasting Serum Glucose and Insulin Level

Fasting serum glucose (FSG) level was measured with the glucose oxidase method[23], fasting serum insulin (FSI) level by competitive radioimmunoassay[24] with an insulin radioimmunoassay kit (Beijing Atom Hightech Co. Ltd., Beijing, China). Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated with FSG and FSI data according to the following formula: HOMA-IR=FSG (mmol/L) × FSI (mIU/L)/22.5[25].

Determination of Hepatic TG and TC Contents

Dried hepatic lipid extract was obtained as previously described[26]. The lipid extract was dissolved in 200 μL dehydrated alcohol for determination of hepatic TG and TC contents as described above.

Determination of FFA Content in Liver and Skeletal Muscle

Tissue homogenate was prepared by homogenizing tissue in an ice-cold Tris-HCl buffer (pH 7.4) (at the ratio of 1/9, w/v). After the homogenate was centrifuged at 3000 rpm for 10 min at 4 ℃, supernatant liquid was obtained. Tissue FFA content in the supernatant was determined immediately as described above.

Analysis of Hepatic Lipase (HL) Activity in Liver, and Lipoprotein Lipase (LPL) in Skeletal Muscle and Adipose Tissue

Tissue homogenate was prepared in an ice-cold buffer containing 0.25 mol/L sucrose, 50 mmol/L Tris-HCl and 80 IU/mL heparin (pH 8.5). After centrifugation at 3000 rpm for 10 min at 4 ℃, the supernatant was reserved. The activit of HL and LPL was determined as previously described[27]. The activity of lipases in tissue was defined as the amount of FFA produced by one milligram tissue protein in the reaction within an hour (FFA μmol/mg protein/hour), and lipase activity was calculated according to the following formula:

Gross activity = $(OD_{test}/OD_{standard}) \times FFA\ concentration_{standard} \times sample\ volume \times 2$

HL activity = $(OD_{inhibited} / OD_{standard}) \times FFA\ concentration_{standard} \times sample\ volume \times 2$

LPL activity = Gross activity-HL activity

Detection of mRNA Expression of PPAR-α in Liver by RT-PCR

Total RNA was extracted from the liver with Trizol reagent (Invitrogen, USA), following its manufacturer's instructions. First-strand cDNA was generated from 3 μg of the total RNA in a volume of 20 μL, using a reverse-transcription kit (Promega, USA) with Oligo dT as a primer. PCR was performed in a final volume of 25 μL containing 2 μg cDNA, 9.5 μL double distilled water, 12.5 μL PCR master mix (Tian-gen Biotechnology, China), 0.5 μL each of the primers (10 pmol/μL) specific for PPAR-α or β-actin (internal control). Primers (PPAR-α: sense primer: 5′-ATG TCC GTG GAG ACC GTC AC-3′; antisense primer: 5′-GGT TCT TAA GGA ACT CGC GTG-3′; β-actin: sense primer: 5′-GAT GGA GTT GAA TGT AGT TT-3′; antisense primer: 5′-CCT GTA TGC CTC TGG TCG TA-3′) were designed using primer 5 and were synthesized by AuGCT Biotechnology (China). PPAR-α was amplified with an initial denaturation at 94 ℃ for 5 min, followed by 32 cycles, with each cycle consisting of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 40 s in GeneAmp PCR system 9700 (PE, USA). As an internal control, β-actin was amplified concomitantly under the same amplification conditions. PCR products (5 μL) were electrophoresed on a 2.0% agarose gel. Band intensity was quantified under UV light using the Sygene Bio-ID system (USA). mRNA expression level was expressed as the ratio of band intensity of the target gene relative to that of β-actin.

Detection of PPAR-α Protein Expression in Liver by Western Blot

Total protein was isolated from liver tissue by homogenization with RIPA lysis buffer containing 50 mmol/L Tris-HCl at pH 7.4, 1% NP-40; 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L PMSF. Protein concentration was determined as previously described[28]. A total of 50 μg protein was first stacked on a 5% SDS-PAGE gel at 80 V, fractionated on a 10% SDS-PAGE gel at 120 V using Bio-Rad mini protein II system, and transferred to a PVDF membrane. Then, the membrane was blocked for 2 h at room temperature with 5% skimmed milk powder in TBST (20 mmol/L Tris-HCl at pH 7.4, 150 mmol/L NaCl, 0.05% Tween-20) to reduce non-specific binding. Primary antibody blotting was incubated overnight at 4 ℃ with goat polyclonal antibody against PPAR-α (Santa Cruz Biotechnology, USA) diluted at 1:5000 in TBST. The membrane was washed and incubated at room temperature for 2 h with rat anti-goat IgG-horseradish

peroxidase secondary antibody diluted at 1:10 000 dilution in TBST (Santa Cruz). Immunoreactivity was determined using an ECL chemoilluminescence reaction kit (Amersham, Arlington Heights, IL, USA) with X-ray film (Kodak, USA). Density quantification of the results was performed using the Sygene Bio-1D system (USA). PPAR-α protein expression was normalized to β-actin. The stripped membrane was re-hybridized with rabbit polyclonal antibody against β-actin diluted at 1:500 (Santa Cruz), and re-incubated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody diluted at 1:5000, (Santa Cruz) following the same procedure as described above.

Statistical Analysis

All data were expressed as $(\bar{x} \pm s)$. Statistical analysis was performed with SPSS 11.0. T test for independent-samples was used to compare differences between the two groups. $P<0.05$ was considered statistically significant.

RESULTS

Effect of CGA on Body Weight in Golden Hamsters on High Fat Diet

At the beginning of the treatment there was no statistical difference between the control and CGA-treated animals in body weight. After 8 weeks, body weight, body weight gain range, and visceral obesity extent of the CGA-treated hamsters were all found to have been significantly lower than those of the control ($P<0.01$) (Table 2).

TABLE 2

Effect of CGA on Body Weight of Golden Hamsters on High Fat Diet ($\bar{x} \pm s$)

Items	Control Group (n=10)	CGA Treatment Group (n=10)	t Test for Independent Samples
Initial Body Weight (g)	81.30 ±1.67	79.90 ±1.57	t=1.019 P=0.321
Final Body Weight (g)	154.20 ±4.67	135.00 ±4.85**	t=3.162 P=0.005
Body Weight Gain (g)	72.90 ±3.39	55.10 ±3.83**	t=3.290 P=0.004
Visceral Adipose (g/100g body weight)	4.34 ±0.27	2.99 ±0.20**	t=4.259 P＜0.001

Note. **$P<0.01$ *vs* control group.

Effect of CGA on Serum Lipid, Glucose, and Insulin Levels in Golden Hamsters on High Fat Diet

As shown in Table 3, at the end of week 8, the serum TG, TC, LDL-C, HDL-C, and FFA levels in CGA treatment group were decreased by 28% ($P<0.05$), 18% ($P<0.01$), 54% ($P<0.01$), 33% ($P<0.01$), and 38% ($P<0.05$), respectively, compared with those in control group. At the same time, the FSG and FSI in CGA treatement group were reduced by 34% ($P<0.05$) and 53% ($P<0.01$), whereas the HOMA-IR in CGA treatment group was elevated by 69% ($P<0.01$) compared with that in control group.

Effect of CGA on Tissue Lipid Content in Golden Hamsters on High Fat Diet

After eight weeks, the content of hepatic TG was lower 38% ($P<0.01$), TC higher 39% ($P<0.01$), and the level of FFA was higher than 26% ($P<0.05$) in CGA treatment group than in control group. No difference was found in hepatic FFA level between the two groups (Table 3).

Effect of CGA on Activity of Tissue Lipase in Golden Hamsters on High Fat Diet

Compared with the control group, the activity of HL in liver was 25% higher ($P<0.05$), and the activity of LPL in skeletal muscle was 79% lower ($P<0.01$) in CGA treatment group than in control group, while there was no significant difference in LPL activity between the two groups (Table 3).

Effect of CGA on mRNA and Protein Expression of PPAR-α in Liver of Golden Hamsters on High Fat Diet

As shown in Figs. 1 and 2, CGA treatment significantly up-regulated the expression of PPAR-α mRNA ($P<0.01$) and protein ($P<0.05$) in the liver of golden hamsters on high fat diet.

TABLE 3

Effect of CGA Treatment on Serum Biochemical Items, Tissue Lipid Contents, and Tissue Lipase Activity in Golden Hamsters on High Fat Diet ($\bar{x} \pm s$)

Items	Control Group (n=10)	CGA Treatment Group (n=10)	t Test for Independent Samples
Serum			
TG (mmol/L)	1.58±0.18	1.14±0.08*	t=2.245 P=0.044
TC (mmol/L)	3.89±0.18	3.20±0.15**	t=2.912 P=0.012
LDL-C (mmol/L)	1.37±0.07	0.63±0.1**	t=6.209 P<0.001
HDL-C (mmol/L)	0.98±0.04	0.66±0.05**	t=4.643 P<0.001
FFA (μmol/L)	1572.52±190.14	978.63±74.23*	t=2.910 P=0.011
FSG (mmol/L)	14.80 ±2.04	9.80±0.44*	t=2.398 P=0.041
FSI (mIU/L)	32.74±2.53	15.51±1.43**	t=6.163 P<0.001
HOMA-IR	22.04±2.28	6.94±0.57**	t=6.409 P<0.001
Liver			
TG (mmol/L)	4.40±0.66	2.72±0.29**	t=2.339 P=0.043
TC (mmol/L)	3.77±0.30	5.25±0.53**	t=-2.415 P=0.033
FFA (μmol/L)	792.38±83.66	771.13±60.42	t=0.124 P=0.903
HL Activity (U/mg protein)	0.09±0.001	0.12±0.01*	t=-2.714 P=0.026
Skeletal Muscle			
FFA (μmol/L)	107.00±6.34	79.25±9.65*	t=-2.404 P=0.031
LPL Activity (U/mg protein)	2.22±0.46	0.46±0.03**	t=-3.816 P=0.006
Visceral Adipose			
LPL Activity (U/mg protein)	2.76±0.25	2.52±0.17	t=-0.783 P=0.448

Note. TG: triglyceride; TC: total cholesterol; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol; FFA: free fatty acid; FSG: fasting serum glucose; FSI: fasting serum insulin; HOMA-IR: homeostatic model assessment-insulin resistance; HL: hepatic lipase; LPL: lipoprotein lipase. *P<0.05, **P<0.01 *vs* control group.

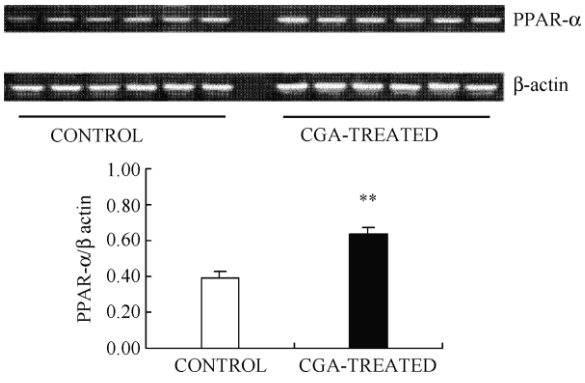

FIG. 1. Effect of CGA on Expression of PPAR-α mRNA in Liver of Golden Hamsters on High Fat Diet in Control Group and CGA Treatment Group. All data are expressed as $\bar{x} \pm s$. $^{**}P<0.01$ *vs* control group.

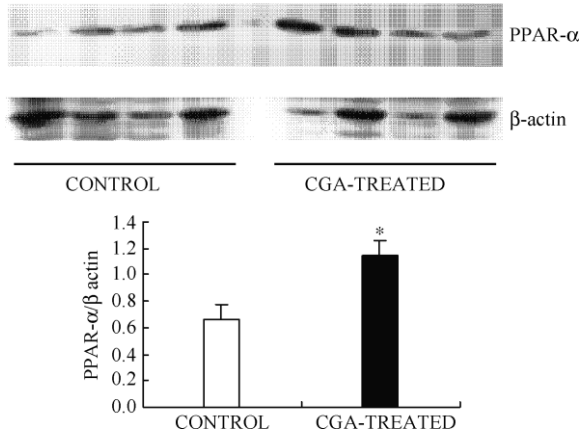

FIG. 2. Effect of CGA on PPAR-α Protein Expression in Liver of Golden Hamsters on High Fat Diet in Control Group and CGA Treatment Group. All data are expressed as $\bar{x} \pm s$. $^{*}P<0.05$ *vs* control group.

DISCUSSION

As a kind of phenolic acids, CGA has been simply highlighted for its antioxidative and antimutagenic bioactivity in the past several decades. Although its *in vitro* inhibitory effects on intestinal glucose uptake and hepatic glucose 6-phosphatase activity have been identified nearly 10 or 20 years before[29-30], only a few *in vivo* studies about the modulating role of CGA in are available, and its underlying mechanism remains uncertain.

In this study, CGA significantly inhibited progress of central obesity, decreased serum lipid and glucose level, increased insulin sensitivity and modified lipid metabolism in the liver and skeletal muscle of golden hamsters on high fat diet. Furthermore, it increased hepatic PPAR-α mRNA and protein expression, which might be associated with the core mechanism of the aforesaid phenomena.

At beginning of the study, no significant difference was found in the initial body weight of the two groups. However, from the first week of intervention, the body weight was significant higher in CGA treatment group than in control group. At end of the study, central obesity of the CGA-treated group was found 31% lower than that of the control group. Similar effects were also reported in previous work of other investigators. In the mice on standard normal diet, oral administration of CGA (60 mg/kg/day for 14 days) showed a tendency to reduce visceral fat and body weight[12]. In Zucker (fa/fa) rats on normal diet, intravenous infusion of CGA (5 mg/kg/day for 3 weeks) decreased body weight[10]. It was reported that CGA does not influence the daily food intake of animals[10], suggesting that CGA exerts it's effect on body weight by promoting metabolism and utilization of fat *in vivo*. After absorbed into blood, dietary fat is packaged inside chylomicra (CM) and transported to extra-hepatic tissues such as muscles and adipose tissue for disposal. A large amount of exogenous TG in these tissues is hydrolyzed by LPL into FFA for energy production or fat deposition. Remnant CM is then cleared in liver. In the present study, no elevated LPL activity was observed in extra-hepatic tissues of the CGA-treated animals (LPL activity of muscles even got reduced), indicating that excessive exogenous fat might flow into the liver. Thus, CGA may act on fat metabolism in the liver, and reduce serum and hepatic TG in animals. However, its mechanism should be further investigated. In this study, we further examined the activity of HL, a key enzyme responsible for exogenous TG hydrolysis in the liver, and found that it was significantly higher in the CGA-treated animals than in the controls. Generally, a large amount of FFA will release into blood due to a high activity of HL. In our study, serum FFA level in CGA-treated animals was reduced and liver FFA level tended to decrease, indicating that an excessive amount of FFA caused by overload of diet fat and hepatic TG hydrolysis can be successfully drained from liver in the presence of CGA.

Increased drainage of FFA can relieve the inhibition pressure of FFA on glucose uptake and oxidation in liver or in muscles, which might induce the hypoglycemic effect of CGA[31]. CGA can improve glucose tolerance and insulin sensitivity, thus promoting some cellular mechanisms in liver, such as increasing hepatic protein and DNA concentrations[10-12]. In the present study, CGA also

showed hypoglycemic and insulin sensitizing action by decreasing FSG, FSI, and HOMA-IR because of the successful clearance of TG and FFA in the liver by CGA. High serum FFA originating from fat rich diet is a main inducer of insulin resistance by decreasing hepatic insulin extraction, enhancing hepatic gluconeogenesis, and competing with glucose as an energy substrate in skeletal muscle[31-33]. In the CGA-treated animals, with a large amount of dietary and TG-derived FFA thoroughly cleared in the liver, little excessive FFA was spared and transported to ultra-hepatic tissues, such as skeletal muscle, the largest organ of glucose metabolism. Furthermore, with activity of LPL in skeletal muscles downregulated by CGA, a less amount of endogenous FFA was produced. A relatively lower level of both exogenous and endogenous FFA in skeletal muscle leads to a less impairment on insulin sensitivity[34]. Accordingly, utilization of glucose as an energy substrate can decrease glucose and insulin level in circulation due to the hypoglycemic effect of CGA.

After going through β-oxidation in mitochondria of hepatic cells, FFA turns into acetyl-CoA and then enters tricarboxylic acid cycle for further catabolism, suggesting that thorough clearance of FFA from the liver might be attributed to the activation of certain upstream regulators of energy metabolism. We examined the effect of CGA on expression of PPAR-α mRNA and protein level in liver, and found that PPAR-α was predominantly expressed in tissues that metabolize a high amount of FFA[35]. PPAR-α modulates transcription of proteins involved in synthesis, transport, and β-oxidation of FFA[36]. Since PPAR-α can enhance β-oxidation of FFA, facilitate catabolism of TG and suppress FFA synthase activity, its agonists have been successfully used in treatment of dyslipidemia[37-38]. In the present study, the expression of PPAR-α mRNA and protein was significantly elevated by CGA, with hepatic HL activity strengthened, serum and hepatic TG and FFA content reduced, indicating that CGA can reduce serum and hepatic fat content through PPAR-α facilitated hepatic FFA drainage pathway[31]. However, whether CGA acts as a direct activator of PPAR-α, or induces its activation through other indirect pathways needs to be clarified.

In this study, CGA also showed its effect on hypocholesteremia. Serum TC and LDL-C were reduced 18% and 54%, respectively, while liver TC was increased 39% in the CGA treatment group, suggesting that CGA can strengthen the ability to dispose cholesterol in the liver. Similar effects of CGA on hepatic TC have also observed in SD rats on high fat diet[39]. Moreover, serum lipid profile was improved by CGA, a paradoxical reduction in HDL-C was also observed in this study. Similar paradoxical findings have been reported in several clinical cases after treatment with fibrate (PPAR-α agonist) or with TZDs (PPAR-γ agonist) or statin (inhibitor of HMG-CoA reducase) in combination[40-43]. The underlying mechanism remains unclear, but this phenomenon suggests that CGA can act on the agonist of PPAR-α.

In conclusion, CGA can improve lipid and glucose metabolism in golden hamsters on high fat diet by strengthening TG and FFA clearance in the liver through activation of PPAR-α. CGA may be used in the prevention and treatment of chronic metabolic diseases, such as metabolic syndrome and type 2 diabetes.

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