Chlorogenic acid from honeysuckle improves hepatic lipid dysregulation and modulates hepatic fatty acid composition in rats with chronic endotoxin infusion

Yan Zhou,1 Zheng Ruan,1,*, Yanmei Wen,1 Yuhui Yang,1 Shumei Mi,1 Lili Zhou,1 Xin Wu,1,2 Sheng Ding,3 Zeyuan Deng,1 Guoyao Wu4 and Yulong Yin1,2

1State Key Laboratory of Food Science and Technology and College of Food Science, Nanchang University, Nanchang 330047, China
2Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China
3Institute of Nutrition and Food Safety, Center for Disease Control and Prevention of Jiangxi Province, Nanchang 330029, China
4Department of Animal Science, Faculty of Nutrition, Texas A&M University, College Station 77843-2471, USA

(Received 5 November, 2014; Accepted 30 June, 2015; Published online 20 January, 2016)

Chlorogenic acid as a natural hydroxycinnamic acid has protective effect for liver. Endotoxin induced metabolic disorder, such as lipid dysregulation and hyperlipidemia. In this study, we investigated the effect of chlorogenic acid in rats with chronic endotoxin infusion. The Sprague-Dawley rats with lipid metabolic disorder (LD group) were intraperitoneally injected endotoxin. And the rats of chlorogenic acid-LD group were daily received chlorogenic acid by intragastric administration. In chlorogenic acid-LD group, the area of visceral adipocyte was decreased and liver injury was ameliorated, as compared to LD group. In chlorogenic acid-LD group, serum triglycerides, free fatty acids, hepatic triglycerides and cholesterol were decreased, the proportion of C20:1, C24:1 and C18:3n-3 acid was increased compared to the LD group. Moreover, levels of phosphorylated AMP-activated protein kinase, carnitine palmitoyltransferase-I, and fatty acid β-oxidation were increased in chlorogenic acid-LD group compared to LD rats, whereas levels of fatty acid synthase and acetyl-CoA carboxylase were decreased. These findings demonstrate that chlorogenic acid effectively improves hepatic lipid dysregulation in rats by regulating fatty acid metabolism enzymes, stimulating AMP-activated protein kinase activation, and modulating levels of hepatic fatty acids.

Key Words: chlorogenic acid, lipid metabolism, fatty acids, AMP-activated protein kinase

Lipid metabolic disorder is a common characteristic of metabolic syndrome and is present at an alarming rate worldwide. It can result in an increased risk of many serious diseases, including obesity, hyperlipidemia, and nonalcoholic fatty liver disease. Lipid accumulation in liver can occur either through increased uptake of fatty acids, increased synthesis within the tissue involved, or reduced fatty acid oxidation/disposal. Therefore, prevention and treatment of fatty acid metabolism is relevant to health promotion.

The balance between lipogenesis and lipolysis is disturbed by various factors, such as endotoxin. Endotoxin has been identified as a strong inducer of nonalcoholic hepatic steatosis and dyslipidemia. The intestine and blood are constantly exposed to various levels of endotoxin because there is more than 1 g of endotoxin presented in the gut, and endotoxin is a toxic component of cell walls of gram-negative bacteria. Feeding with a high fat diet (approximately 900 kcal) increased endotoxin in serum by approximately 50% in healthy persons. Diet can induce alteration of gut microbiota and increased levels of endotoxin, and then increase the permeability of the intestine in which endotoxin permeates into the blood and liver. Disrupted intestinal epithelium integrity led to increased portal endotoxemia and exposure of the liver to high levels of endotoxin. Hence, humans are continuously exposed to low doses of endotoxin, and remission of endotoxin-induced metabolism disorder, such as lipid metabolic disorder, is important to health.

Nutritional supplements, which are extracted from plants, are extremely popular on regulation metabolism disorders. Honeysuckle is the flower of the plant, which belongs to the family Lonicera caerulea L., is widely harvested in China, Japan, and Europe. It is consumed as herbal tea and medicine in China and Japan. The pharmacological activities of honeysuckle include anti-inflammatory, anti-atherogenic, and anti-carcinogenic effects. Chlorogenic acid (CGA) is considered to be one of the major components of honeysuckle, and has multiple physiological functions. CGA regulates glucose homeostasis by inhibiting glucose-6-phosphatase activity, decreasing intestinal glucose absorption, modulating glucose release in the liver, and increasing glucose uptake in muscle tissue. CGA also has shown anti-lipogenesis effects and improves plasma lipid profiles in obesity animals. It decreased the blood triglyceride and cholesterol, lowered lipid profiles in liver, epididymal adipose tissue and heart in high fat induced obese mice. Fatty acid metabolism in liver is subject to extensive in vivo regulation, in particular by the control of fatty acid entry into the cell, transfer of fatty acids into the mitochondria and the capacity of the β-oxidation. The balance between the uptake and utilization of fatty acid will ultimately determine the magnitude of lipid accumulation in liver cells. However, there has been no scientific literature available on the effect of CGA supplementation on fatty acid composition.

Fatty acid metabolism is a complex process involved with fatty acid synthesis and fatty acid oxidation. AMP-dependent protein kinase (AMPK) plays a key role in fatty acid metabolism, activation of AMPK affects key enzymes in fatty acid synthesis, in

*To whom correspondence should be addressed.
E-mail: ruanzheng@ncu.edu.cn
which acetyl-CoA carboxylase (ACC) activity is inhibited and fatty acid synthase (FAS) expression is decreased. Activation of AMPK not only inhibits fatty acid synthesis but also activates fatty acid β-oxidation by reducing the levels of malonyl-CoA as the product of ACC. In vitro study, Tsuda et al. reported that no effects of CGA treatment on AMPK activation were observed in rat skeletal muscle, while Ou et al. demonstrated that CGA stimulated glucose uptake in L6 myoblasts through AMPK. In vivo study, chronic administration of CGA stimulated AMPK activation in liver in Lepr db/db mice. However, Mubarak et al. reported that CGA supplementation in a high fat diet inhibited AMPK activation in obese C57BL/6 mice. Thus, further evidences should be provided on the effect of CGA on AMPK.

In the present study, we investigated the effect of CGA (extract from honeysuckle) on lipid metabolic disorder in rats induced by low-dose endotoxin infusion. Thus, the growth parameters, organ weights, adipose and liver tissue histology, serum and hepatic lipid parameters were determined, we also analyzed the influence of CGA on fatty acid composition and enzymes involved in lipogenesis and fatty acid oxidation.

Materials and Methods

Animals, diets and experimental design. The experiment was conducted with thirty-two female Sprague-Dawley rats aged 6 weeks (182.84 g (SE 2.13)) purchased from Changsha Tianxin Biotechnology Co., Ltd. (Changsha, China). The rats aged 6 week were before sexual maturation period. Hence, all rats were at same starting line of estrous cycle would not affect the accuracy of experimental results. The experiment was approved by the Nanchang University Animal Care and Use Committee. The rats were individually housed in an air-conditioned room (22 ± 2°C) with a 12-h light:12-h dark cycle in the Animal Laboratory of Jiangxi Province Center for Disease Control and Prevention (Nanchang, China).

After a 7-days adaptation period, rats were randomly distributed in four experimental groups (eight animals each group), with free access to water and a standard laboratory diet (China General Quality Standards for Animal Feed, GB14924.1-2001). CGA from honeysuckle (≥98% purify, Shanghai, China) was dissolved in sterile saline and orally administered into the rats every day for 28 days between 09:00 am and 10:00 am. Endotoxin (Escherichia coli O55:B5, Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and orally administered into the rats every day for 28 days between 09:00 am and 10:00 am. Endotoxin (Escherichia coli O55:B5, Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline.

The rats of normal group were daily received sterile saline by intragastric administration (ig) and sterile saline by intraperitoneal injection (ip). The rats of CGA group were daily received chlorogenic acid (60 mg CGA/kg body weight) by ig and sterile saline by ip. The rats with lipid metabolic disorder (LD group) were daily received sterile saline by ig and intraperitoneally injected endotoxin (300 μg/kg body weight). The CGA-LD group was daily administered with CGA at dosage of 60 mg/kg body weight by ig and intraperitoneally injected endotoxin (300 μg/kg body weight). At the end of the experimental period and after a fasting period of 12 h, animals were sacrificed by cardiac exsanguination under anesthesia by using an intraperitoneal injection of an overdose (45 mg/kg) of sodium pentobarbital. Liver, visceral adipose tissue, kidney, spleen, and intestine samples were harvested, weighed and immediately frozen.

Growth and serum biochemical parameters. The body weight and food intake of rats were measured weekly. The food efficiency ratio (FER) was calculated as body weight gain in gram divided by food intake in gram. Blood samples were collected from rats for the measurement of serum levels of triglyceride, total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and free fatty acids. Liver injury was assessed by measurement of total bilirubin, alanine transaminase (ALT) and aspartate aminotransferase (AST).

Briefly, blood samples were centrifuged at 4,000 × g for 15 min at 4°C, and the supernatant fluid (serum) was obtained. The kits of triglyceride, total cholesterol, HDL-C, LDL-C, AST, and ALT were purchased from Leadman Company (Beijing, China). Serum biochemical parameters were measured by a biochemical analyzer (Beckman Coulter Inc., Fullerton, CA).

The analysis of bilirubin and free fatty acid were determined by the spectrophotometric method using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Hematoxylin and eosin staining. Histology analyses of liver and visceral adipose tissue were measured by hematoxylin and eosin staining (H&E) according to methods described previously. At the end of the experiment, liver and visceral adipose tissue were dissected and fixed in 10% phosphate-buffer formalin overnight before being incubated in 50% ethanol (v/v) and then promptly embedded with paraffin. The tissues were then cut into 4 μm sections and stained with hematoxylin and eosin to reveal the structural features, and observed under a light microscope. The Motic Med 6.0 (Beijing, China) and the Image-Pro Plus 6.0 were used to examine these sections with a final magnification of ×200. The number of adipocytes per microscopic field (density) was determined by the spectrophotometric method using a commercial kit (Nanjing Jiancheng Bioengineering Institute).

Lipid regulating enzymes activities and content. For lipogenic enzyme analysis, samples of liver (500 mg) were homogenized in 5 ml buffer (pH 7.6) containing 0.02 mol/L ethylene diamine tetraacetic acid (EDTA) and 0.002 mol/L dithiothreitol (DTT). After centrifugation at 1,000 × g for 15 min, the lower clear organic phase solution was transferred into a new glass tube and the lipid fraction was dried under a nitrogen stream. Then, the lyophilized powder was dissolved in hexane as the liver lipid extract. The liver triglyceride and cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute).

Lipid regulating enzymes activities and content. For lipogenic enzyme analysis, samples of liver (500 mg) were homogenized in 5 ml buffer (pH 7.6) containing 0.02 mol/L ethylene diamine tetraacetic acid (EDTA) and 0.002 mol/L dithiothreitol (DTT). After centrifugation at 1,000 × g for 15 min, the lower clear organic phase solution was transferred into a new glass tube and the lipid fraction was dried under a nitrogen stream. Then, the lyophilized powder was dissolved in hexane as the liver lipid extract. The liver triglyceride and cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute).

Determination of triglyceride and cholesterol in the liver. Liver lipids were extracted as described previously. Briefly, liver (1 g) was homogenized with a chloroform/methanol/distilled water (2:2:1) mixture. After centrifugation (10,000 × g for 15 min), the lower clear organic phase solution was transferred into a new glass tube and the lipid fraction was dried under a nitrogen stream. Then, the lyophilized powder was dissolved in hexane as the liver lipid extract. The liver triglyceride and cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute).

Lipid regulating enzymes activities and content. For lipogenic enzyme analysis, samples of liver (500 mg) were homogenized in 5 ml buffer (pH 7.6) containing 0.02 mol/L ethylene diamine tetraacetic acid (EDTA) and 0.002 mol/L dithiothreitol (DTT). After centrifugation at 1,000 × g for 15 min, the lower clear organic phase solution was transferred into a new glass tube and the lipid fraction was dried under a nitrogen stream. Then, the lyophilized powder was dissolved in hexane as the liver lipid extract. The liver triglyceride and cholesterol in the lipid extracts were measured by enzymatic colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute).
by Bieber et al. with minor modifications. Briefly, the assay was conducted at 37°C for 2 min and was initiated by the addition of 50 µl of mitochondrial suspension to 950 µl of the following standard reaction medium: 116 mM/L Tris-HCl (pH 8.0), 1.1 mM/L EDTA, 0.5 mM/L dithionitrobenzoic acid, 0.2% Triton X-100, 75 mM/L palmitoyl-CoA, 2.5 mM/L carnitine. The change in absorbance at 412 nm was measured, and the activity was expressed as nmol CoASH/min/mg protein.

Fatty acid β-oxidation activity was measured from the final product of NADH using palmitoyl substrates. The assay mixture contained 50 mM/L Tris-HCl (pH 8.0), 10 mM/L β-mercaptoethanol, 1.5% BSA (1.5 g/100 ml), 20 mM/L NAD+, 2% Triton X-100 (2 g/100 ml), 1 mM/L FAD, 100 mM/L KCN and 5 mM/L palmitoyl-CoA. The reaction was initiated by the addition of 0.1 ml of mitochondrial suspension, and incubated at 37°C for 5 min. The absorption value at 340 nm was measured.

The content of FAS, ACC and CPT-1 was measured with ELISA according to manufacturer’s protocol (Cusabio, Wuhan, China). The ELISA microplate was read using an ELISA reader (Dynatech Laboratories, Chantilly, VA) with a maximum absorbance of 450 nm.

**Hepatic fatty acid composition.** The fatty acid composition of the liver was determined by capillary gas chromatography. The lipid extracts from liver were transmethylated in the presence of 2% sodium methylate/methanol at 37°C for 20 min. The samples were cooled at ~20°C for 10 min, and 60 µl 0.1 mol/L oxalic acid was added. After centrifugation, the upper phase was obtained for fatty acid analysis by an Agilent 7890A gas chromatogram (Agilent Technologies, Palo Alto, CA) with a flame ionization detector using a capillary column (100 m × 0.25 µm × 0.20 µm, Supelco, Bellefonte, PA). The temperature program was as follows: 140°C initial temperature for 5 min, 4°C/min to 240°C, 1 min at this temperature and, thereafter temperatures were 260°C, respectively. The carrier gas was hydrogen at a flow rate of 30 ml/min. Individual fatty acid peaks were identified by comparison of their retention times with those of standards. The relative amount of each fatty acid (% of total fatty acid) was calculated by integrating the area under the peak and dividing the result by the total area for all fatty acids.

**Table 1.** Body weight, body weight gain and food intake in rat supplemented with CGA

| Body weight gain | Normal | CGA | LD | CGA-LD |
|------------------|--------|-----|----|--------|
| D1–D7 (g/d)      | 3.07 ± 0.19* | 3.28 ± 0.49* | 3.08 ± 0.36* | 2.51 ± 0.52* |
| D8–D14 (g/d)     | 3.19 ± 0.28* | 3.89 ± 0.67* | 4.05 ± 0.94* | 3.29 ± 0.38* |
| D15–D21 (g/d)    | 2.79 ± 0.23* | 3.03 ± 0.38* | 4.63 ± 0.33b | 3.18 ± 0.28* |
| D22–D28 (g/d)    | 1.90 ± 0.15a | 2.10 ± 0.13a | 3.54 ± 0.18a | 2.25 ± 0.28a |
| Total body weight gain (g) | 74.00 ± 4.85* | 82.33 ± 6.08b | 102.00 ± 7.46a | 73.20 ± 7.08a |

**Food intake**

| Food intake | Normal | CGA | LD | CGA-LD |
|-------------|--------|-----|----|--------|
| D1–D7 (g/d) | 17.79 ± 1.27a | 18.50 ± 0.85a | 17.88 ± 0.52a | 16.69 ± 0.77a |
| D8–D14 (g/d) | 19.86 ± 0.85a | 22.82 ± 1.40a | 21.59 ± 1.36a | 20.90 ± 1.19a |
| D15–D21 (g/d) | 18.26 ± 0.42a | 19.93 ± 1.38a | 18.23 ± 2.02b | 14.98 ± 0.41a |
| D22–D28 (g/d) | 18.37 ± 0.79a | 16.53 ± 1.13a | 16.22 ± 1.06a | 16.24 ± 0.99a |
| Total food intake (g) | 522.33 ± 25.24a | 548.13 ± 19.02a | 520.96 ± 20.00a | 492.20 ± 21.24a |

**Food efficiency ratio (g body weight gain/g food intake)**

| Food efficiency ratio | Normal | CGA | LD | CGA-LD |
|-----------------------|--------|-----|----|--------|
| D1–D7                 | 0.17 ± 0.01a | 0.18 ± 0.01a | 0.18 ± 0.1a | 0.15 ± 0.01a |
| D8–D14                | 0.17 ± 0.02a | 0.17 ± 0.01a | 0.19 ± 0.03a | 0.16 ± 0.01a |
| D15–D21               | 0.16 ± 0.01a | 0.16 ± 0.02a | 0.26 ± 0.03a | 0.22 ± 0.02b |
| D22–D28               | 0.11 ± 0.01a | 0.13 ± 0.01a | 0.23 ± 0.03a | 0.14 ± 0.01a |
| D1–D28                | 0.14 ± 0.01a | 0.15 ± 0.01a | 0.20 ± 0.01a | 0.15 ± 0.01a |

Values are expressed as the mean ± SE, n = 8. The values with different lowercase superscript letters in the same line are significantly different (p<0.05), the values with different uppercase superscript letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey’s test.

**Western blot analysis of AMPK and p-AMPK.** Total liver protein was isolated by phosphorylated protein extraction kits including phosphatase inhibitors and a protease inhibitor. The protein content on the lysates was estimated by the coomassie brilliant blue method. Proteins (80 µg) were separated with 10% SDS-PAGE and then transferred to a PVDF membrane (2 h, 200 mA). The membranes were blocked in 5% BSA for 1 h at room temperature. Then, the membranes were incubated overnight with a primary antibody against AMPK (1:1,000, Cell Signaling Technology, Beverly, MA) and Thr172-phosphorylated AMPK (1:1,000, Cell Signaling Technology) in blocking buffer. After washing in Tris-buffered saline/Tween 20 under gentle agitation, the membranes were incubated for 1 h with a horseradish peroxidase-labelled anti-rat IgG (1:10,000). After further washing, blots were treated with enhanced chemiluminescence detection reagents. Blot intensities were measured using Image J software (NIH).

**Statistical analysis.** All data are presented as the mean ± SE of the four different groups. Differences between variants were analyzed by analysis of variance (ANOVA) and Tukey’s test, using SPSS 17.0. Significance levels at p<0.05 were considered to indicate statistical significance.

**Results**

**CGA reduced body weight gain without affecting food intake.** The body weight gain, food intake, and FER were measured during 4 weeks as shown in Table 1. The body weight gain during the third week (D15–D21, p<0.05), forth week (D22–D28, p<0.01), and total body weight gain (p<0.05) were significantly increased in LD group, as compared to the normal group. Meanwhile, compared to LD group, the body weight gain in the third week (D15–D21, p<0.05) and forth week (D22–D28, p<0.01), and total body weight gain (p<0.05) week were significantly decreased.
in CGA-LD group.

The significant difference on food intake was only found between normal group and CGA-LD group, and between CGA group and CGA-LD group ($p<0.05$). Compared to normal group, the FER in LD group were increased in the experimental time (D15–21, 22–28, and D1–D28). Compared to LD group, the FER in CGA-LD group were decreased in the experimental time (D22–28 and D1–28).

**CGA reduced tissue weight and area of adipocytes.** The relative tissue weight of liver ($p<0.05$), visceral adipose ($p<0.05$), spleen ($p<0.05$) and thymus ($p<0.05$) were significantly decreased in the CGA-LD group compared to the LD group, and especially relative visceral adipose weight, which decreased by 43.09% ($p<0.05$, 3.76 g/100 g body weight (SE 0.37) in the LD group vs 2.14 g/100 g body weight (SE 0.23) in the CGA-LD group) (Fig. 1A).

The morphological characteristics of visceral adipose tissue were measured by H&E staining (Fig. 1B–E). The area of adipocytes in LD rats was significantly increased compared to normal rats (Fig. 1F). CGA supplementation inhibited adipocyte hypertrophy (F).

**Fig. 1.** Effect of CGA on relative tissue weights and morphology of adipose tissue. CGA supplementation decreased relative tissue weights (except kidney) (A), compared to the LD group. Values are expressed as the mean ± SE, n = 8. Histological analyses of visceral adipose tissue from normal (B), CGA (C), LD (D), and CGA-LD (E) groups; (n = 3). CGA inhibited adipocyte hypertrophy (F), values are expressed as the mean ± SE, n = 3. Values with different lowercase letters are significantly different ($p<0.05$), values with different uppercase letters are significantly different ($p<0.01$), as analyzed by one-way ANOVA and the Tukey’s test.

Y. Zhou et al.
trophy. The area of adipocytes in CGA-LD group rats significantly decreased compared to LD group rats by 25.24% (p < 0.05, 824.75 ± 54.28 μm² (SE 56.58) in the LD group vs 616.55 ± 45.74 μm² (SE 24.08) in the CGA-LD group) (Fig. 1F).

**CGA alleviated dyslipidemia.** CGA supplementation significantly modulated the levels of serum triglyceride, HDL-C, and free fatty acid compared to the LD group (Table 2). CGA supplementation significantly lowered the level of serum triglyceride (p < 0.01) and serum free fatty acid (p < 0.05) compared with the LD group. HDL-C level was significantly increased in the CGA-LD group compared to the LD group. However, CGA supplementation in the CGA-LD group had no significant effect on total cholesterol or LDL-C.

**CGA relieved liver injury and improved liver morphology.** The liver plays a central role in whole body lipid metabolism. To assess CGA on liver injury the liver morphology, the activity of MPO in the liver and the levels of serum AST, ALT, and bilirubin were measured. Normal liver microstructure in the normal group was shown by H&E staining (Fig. 2E). Comparison with normal rats, LD group rats had a higher infiltration of inflammatory cells (Fig. 2E and G). CGA supplementation inhibited inflammatory cell infiltration compared with the LD group (Fig. 2G and H).

MPO was a marker of lymphocyte infiltration. The activity of MPO (p < 0.01) was significantly decreased in the CGA-LD group compared to the LD group (Fig. 2I). As a consequence of liver dysfunction and injury, LD group rats exhibited elevated levels of bilirubin (p < 0.01), ALT (p < 0.05) and AST (p < 0.05). CGA-LD group rats had significantly reduced levels of bilirubin (p < 0.01), activity of ALT (p < 0.05) and AST (p < 0.05) compared with LD group rats (Fig. 2J–L).

**CGA ameliorated fat accumulation in liver.** Concurrently, CGA supplementation greatly decreased the levels of hepatic triglycerides and cholesterol in the LD group. Staining with Oil-Red O confirmed the presence of lipid droplets (red area) within hepatocytes (Fig. 3A–D). The number and density of lipid droplets in the liver of the CGA-LD group was lower than that of the LD group. Additionally, the levels of liver triglyceride (p < 0.01) and cholesterol (p < 0.01) were decreased in CGA-LD group rats compared to LD group rats (Fig. 3E and F).

**Liver fatty acid composition.** In CGA-LD group, CGA supplementation increased the proportion of C18:3n-3 (p < 0.01), and significantly decreased the proportion of C18:3n-6 (p < 0.05), C20:1 (p < 0.05), and C24:1 (p < 0.05), in comparison with LD group rats (Table 3). Compared with normal rats, no effects on liver fatty acid composition were found in CGA group. The proportions of C18:0 (p < 0.05), C18:2n-6 (p < 0.05) and C18:3n-3 (p < 0.05) in LD group rats liver were decreased compared to the normal group, and the proportions of C15:0 (p < 0.05), C16:0 (p < 0.05), C24:0 (p < 0.05), C20:1, C24:1 (p < 0.01), C18:2n-6, and C18:3n-6 (p < 0.01) were increased compared to normal animals. The desaturase activity indexes of fatty acids in livers were estimated as the ratio of product/precursor of individual fatty acids. A9-18 and Δ6-desaturase activity index were significant increased (p < 0.01) in LD group compared to normal group. A9-18 and Δ6 desaturase activity index in CGA-LD group were significantly decreased, as compared with LD group rats (p < 0.05). No significant differences were found on A9-16 in all treatment groups (Table 3).

**CGA increased fatty acid oxidation, inhibited fat synthesis, and promoted AMPK activation in liver.** CGA supplementation in the CGA-LD group significantly increased mitochondrial fatty acid β-oxidation (79.53%, p < 0.05), CPT-1 content (57.21%, p < 0.05), and CPT-1 activity (46.15%, p < 0.05) in liver compared with the LD group. The content of CPT-1 in the CGA group was significantly increased compared to the normal group. Moreover, compared with the LD group, the content of ACC in the CGA-LD group was significantly decreased by 43.37% (p < 0.01), the content of FAS was lowered by 30.99% (p < 0.05), and the activity of FAS was significantly decreased by 22.81% (p < 0.05) (Table 4).

AMPK plays an important role in regulating lipid metabolism. Recent studies have shown CGA-stimulated AMPK activation in vitro. We hypothesized that the ameliorative effect of CGA on fat accumulation in liver resulted from an increase in hepatic AMPK activation. To test this hypothesis, we investigated the levels of AMPK and the p-AMPK by western blot. CGA supplementation increased the level of p-AMPK (p < 0.01) in the CGA-LD group compared to the LD group (Fig. 4).

**Discussion**

We sought to assess the effect of CGA supplementation on disordered lipid metabolism in rats, the microscopic appearance of liver and visceral adipose tissue, the serum and hepatic lipid parameters, the fatty acid composition in liver, and the signaling pathways associated with lipid metabolism in rats. Our results support the hypothesis that CGA supplementation can improve disordered lipid metabolism. In present study, compared to normal group, body weight gain, organ weight, and FER in LD group were increased, not food intake. These results indicated that chronic endotoxin infusion induced body weight gain compared to normal rats, not by excessive food intake (energy intake). Cani et al. reported that body weight and visceral fat mass was correlated positively with plasma endotoxin levels, and chronic infusion of very low rate of LPS increased body weight without excessive energy intake. Some other articles demonstrated that endotoxin was correlated with obesity or insulin resistance.

The phenomenon (increase of FER and body weight gain induced by endotoxin infusion, no significant variation of food intake) may be explained for two aspects. First, endotoxin infusion decreased energy expenditure and impaired catabolic metabolism of nutrients. It was evidenced by the increase of visceral adipose tissue weight and visceral adipocyte hypertrophy after endotoxin infusion (Fig. 1). Second, the inhibition of AMPK activation (Fig. 4) induced by endotoxin infusion indicated that the catabolic metabolism of nutrients was inhibited. The inhibition of catabolic process could be proved by decrease of fatty acid oxidation (Table 4). Hence, the inhibition of catabolic metabolism promoted protein or fat synthesis (increase of triglycerides and cholesterol content, Fig. 3) and increased body weight. The inhibitory effect of endotoxin on catabolic metabolism, impaired Krebs cycle and mitochondrial respiration activity were evidenced by previous studies.

In previous studies, it had been reported CGA did not affect food intake. In obesity animals (or abnormal physiological state), CGA decreased the body weight, but not food intake.

In LPS-induced acute liver injury rat, CGA injection maintained normal reddish color of the liver and did not affect food intake. The effect of CGA on food intake between CGA and Normal group in our study was almost same, and the food intake between CGA-LD and LD group was no difference. The effect of CGA on food intake was concordance with previous study. However, food intake of CGA-LD group in D15–D21, was significantly different from normal group. The reason for this phenomenon may due to experimetal time and joint action of endotoxin and CGA. First, the peroid of D15–D21 may be the fierce and special period in which the disordered metabolism is serious. Since body weight gain and FER of LD group in 3rd week (D15–21) were significantly increased compared to normal group, and body weight gain and FER of LD group in D1–7 or D8–14 were not significantly increased compared to Normal group. Second, food intake can be affected by the metabolism of glucose and fatty acids. CGA had been reported to increase fatty acid oxidation, inhibit fatty acid synthesis, and decrease glucose intolerance and insulin resistance. However, Mubarak et al. reported the liver of mice...
Table 2. Serum lipids in rats supplemented with CGA

| Serum lipid parameters      | Normal       | CGA          | LD           | CGA-LD        |
|-----------------------------|--------------|--------------|--------------|---------------|
| Triglyceride (mmol/L)       | 0.89 ± 0.08<sup>A</sup> | 1.01 ± 0.06<sup>A</sup> | 1.31 ± 0.10<sup>B</sup> | 0.91 ± 0.04<sup>A</sup> |
| Total cholesterol (mmol/L)  | 2.21 ± 0.10<sup>A</sup> | 2.36 ± 0.15<sup>B</sup> | 2.75 ± 0.12<sup>B</sup> | 2.87 ± 0.14<sup>B</sup> |
| HDL-cholesterol (mmol/L)    | 0.64 ± 0.03<sup>A</sup> | 0.73 ± 0.04<sup>B</sup> | 0.66 ± 0.02<sup>B</sup> | 0.78 ± 0.03<sup>B</sup> |
| LDL-cholesterol (mmol/L)    | 0.80 ± 0.08<sup>A</sup> | 0.75 ± 0.06<sup>A</sup> | 1.41 ± 0.13<sup>B</sup> | 1.52 ± 0.16<sup>B</sup> |
| Free fatty acid (μmol/L)    | 385.09 ± 31.31<sup>A</sup> | 384.50 ± 25.14<sup>A</sup> | 513.34 ± 23.01<sup>B</sup> | 405.19 ± 23.56<sup>A</sup> |

Values are expressed as the mean ± SE, n = 8. The values with different lowercase superscript letters in the same line are significantly different (p<0.05), the values with different uppercase superscript letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey's test.

Fig. 2. CGA reduced the signs of liver pathology. Morphology of livers from normal (A), CGA (B), LD (C), and CGA-LD (D) groups, obtuse liver edge were marked with head arrows. Histological analysis of livers (×200, n = 6): samples from normal (E), CGA (F), LD (G), and CGA-LD (H) groups were stained with H&E staining. Inflammatory cells were marked with arrows. CGA inhibited the increase of MPO activity in liver (I), serum bilirubin content (J), serum ALT (K), and AST (L) activity compared to the LD group. Values are expressed as the mean ± SE, n = 8. Values with different lowercase letters are significantly different (p<0.05), values with different uppercase letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey's test.
fed a high-fat diet supplemented with CGA impaired fatty acid oxidation, and promoted glucose intolerance. These results indicated CGA had bidirectional regulatory effect on metabolism. The number of differences of food intake between CGA-LD and Normal group (D15–21) may be induced by CGA supplements or by metabolic disorders (endotoxin-induced) or by the force of CGA combined with metabolic disorders. However, it need further research for specific period, for example D15–21.

Peritoneal administration of endotoxin for four weeks induced a significant increase in body weight gain, serum triglyceride, cholesterol, LDL-C, and free fatty acids, as well as visceral adipocytes hypertrophy. These results indicated that endotoxin infusion induced lipid metabolic disorder. The same observation in mice has previously been seen by Cani et al. and others. Uchiumi et al. has reported that continuous subcutaneous administration of endotoxin increased serum levels of triglycerides. The liver is the central organ for triglyceride, cholesterol, and lipoprotein metabolism. CGA supplementation ameliorated liver injury and decreased liver lipid content in our study. Through a histological study (H&E staining), CGA supplementation reduced macro- and micro-alterations of hepatic structure in the CGA-LD group. CGA also reduced MPO activity, serum bilirubin content, and AST and ALT activity in the liver, implying that CGA relieved endotoxin-induced liver injury. The number of lipid droplets in the CGA-LD group was reduced when measured by Oil-Red O staining. Triglyceride and cholesterol levels were significantly decreased (Fig. 3). Our results indicated that the intake of CGA for four weeks relieved liver injury and suppressed lipid accumulation in the LD group.

Lipogenic enzymes are essential for the biosynthesis of fatty acids, triglycerides and cholesterol. Decreased activities of these enzymes, such as ACC and FAS, could reduce the availability of fatty acids for the synthesis of hepatic triglycerides. As a result, the esterification of free fatty acids to triglycerides in the liver leads to adipose accumulation that is accelerated by increased lipogenesis as a consequence of decreased fatty acid oxidation increasing availability of fatty acids. CGA inhibited fatty acid synthesis activity in vitro to ameliorate HepG2 lipid accumulation.

![Fig. 3. CGA relieved fat accumulation in liver. Oil Red O staining of live tissue from normal (A), CGA (B), LD (C), and CGA-LD (D) groups. Lipid droplets marked by arrows. The levels of triglycerides (E) and cholesterol (F) in the livers were showed. Values are expressed as the mean ± SE, n = 8. Values with different lowercase letters are significantly different (p<0.05), values with different uppercase letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey’s test. See color figure in the on-line version.](image-url)
Table 3. Liver fatty acid composition in rats supplemented with CGA

| Fatty acid (% of total fatty acid) | Normal | CGA | LD | CGA-LD |
|----------------------------------|--------|-----|----|--------|
| Myristic acid (C14:0)            | 0.44 ± 0.043* | 0.45 ± 0.07* | 0.55 ± 0.05* | 0.54 ± 0.05* |
| Pentadecanoic acid (C15:0)      | 0.23 ± 0.04* | 0.34 ± 0.04* | 0.43 ± 0.06* | 0.38 ± 0.02* |
| Palmitic acid (C16:0)            | 17.96 ± 0.49* | 19.22 ± 0.34* | 19.83 ± 0.38* | 19.49 ± 0.64* |
| Heptadecanoic acid (C17:0)      | 0.37 ± 0.049* | 0.28 ± 0.07* | 0.38 ± 0.09* | 0.18 ± 0.07* |
| Stearic acid (C18:0)             | 25.49 ± 0.91* | 25.01 ± 0.79* | 23.16 ± 0.74* | 22.27 ± 1.05* |
| Arachidic acid (C20:0)           | 0.11 ± 0.01* | 0.10 ± 0.01* | 0.10 ± 0.02* | 0.08 ± 0.02* |
| Behenic acid (C22:0)             | 0.13 ± 0.01* | 0.13 ± 0.01* | 0.15 ± 0.02* | 0.15 ± 0.03* |
| Lignoceric acid (C24:0)          | 0.35 ± 0.04* | 0.37 ± 0.04* | 0.50 ± 0.03* | 0.47 ± 0.04* |
| Myristoleic acid (C14:1)         | 0.16 ± 0.03* | 0.16 ± 0.02* | 0.16 ± 0.01* | 0.17 ± 0.01* |
| Hexadecenolic Acid (C16:1)      | 1.37 ± 0.06* | 1.01 ± 0.18* | 1.34 ± 0.14* | 1.33 ± 0.17* |
| Ginkgolic acid (C17:1)           | 0.14 ± 0.02* | 0.12 ± 0.02* | 0.13 ± 0.02* | 0.11 ± 0.02* |
| Elaidic acid (C18:1-n-9)         | 5.24 ± 0.51* | 5.35 ± 0.57* | 6.87 ± 0.49* | 6.41 ± 0.79* |
| Oleic acid (C18:1-n-9)           | 4.86 ± 0.17* | 4.68 ± 0.17* | 4.86 ± 0.16* | 5.03 ± 0.35* |
| Gadoleic acid (C20:1)            | 0.30 ± 0.024* | 0.35 ± 0.044* | 0.54 ± 0.057* | 0.34 ± 0.054* |
| Nervonic acid (C24:1)            | 0.52 ± 0.010* | 0.50 ± 0.053* | 0.75 ± 0.031* | 0.62 ± 0.012* |
| Linoleic acid (C18:2n-6t)        | 0.17 ± 0.012* | 0.17 ± 0.02* | 0.11 ± 0.012* | 0.11 ± 0.03* |
| Linoleic acid (C18:2n-6c)        | 13.97 ± 0.3*  | 15.00 ± 0.48* | 16.83 ± 0.69* | 15.79 ± 0.26* |
| Eicosadienoic acid (C20:2)       | 0.29 ± 0.018* | 0.29 ± 0.02*  | 0.32 ± 0.02*  | 0.33 ± 0.01*  |
| γ-Linolenic acid (C18:3n-6)      | 0.29 ± 0.044* | 0.31 ± 0.025* | 0.60 ± 0.099* | 0.35 ± 0.046* |
| α-Linolenic acid (C18:3n-3)      | 0.45 ± 0.044* | 0.52 ± 0.074* | 0.18 ± 0.068* | 0.54 ± 0.082* |
| Dihomoγ-linolenic acid (C20:3n-6) | 0.56 ± 0.03*  | 0.56 ± 0.02*  | 0.60 ± 0.02*  | 0.60 ± 0.03*  |
| Arachidonic acid (C20:4n-6)      | 25.13 ± 1.26* | 22.63 ± 1.35* | 24.23 ± 1.77* | 22.57 ± 1.59* |
| Total SFA                        | 45.09 ± 0.69* | 45.70 ± 0.89* | 45.29 ± 0.68* | 43.27 ± 0.38* |
| Total MUFA                       | 13.53 ± 0.93* | 14.41 ± 1.26* | 13.29 ± 0.84* | 15.31 ± 1.14* |
| Total PUFA                       | 40.75 ± 0.55* | 40.73 ± 0.48* | 41.38 ± 0.56* | 40.81 ± 0.39* |
| Desaturase activity index        |          |          |          |          |
| Δ6                               | 0.002 ± 0.002* | 0.021 ± 0.002* | 0.035 ± 0.004* | 0.022 ± 0.003* |
| Δ9-16                            | 0.071 ± 0.003* | 0.052 ± 0.009* | 0.068 ± 0.009* | 0.068 ± 0.009* |
| Δ9-18                            | 0.40 ± 0.034* | 0.40 ± 0.034* | 0.58 ± 0.031* | 0.46 ± 0.014* |

Δ6 is the ratio of C18:3n-6/C18:2n-6. Δ9-16 is the ratio of C16:0/C18:0. Δ9-18 is the ratio of C18:1/C18:0. Values are expressed as the mean ± SE, n = 6. The values with different lowercase superscript letters in the same line are significantly different (p<0.05), values with different uppercase superscript letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey’s test.

Table 4. The content and activity of lipid-regulating enzymes in rats supplemented with CGA

| Lipid-regulating Enzymes Content                      | Normal          | CGA             | LD              | CGA-LD          |
|------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| CPT-1 (ng/l g mitochondrial protein)                 | 52.98 ± 2.98*   | 63.73 ± 5.67*   | 28.98 ± 3.16*   | 45.56 ± 3.11*   |
| ACC (pmol/g protein)                                 | 3.28 ± 0.35*    | 3.03 ± 0.47*    | 8.97 ± 0.83*    | 5.08 ± 0.73*    |
| FAS (nmol/g protein)                                 | 0.48 ± 0.03*    | 0.51 ± 0.04*    | 0.71 ± 0.05*    | 0.49 ± 0.04*    |

| Lipid-regulating Enzymes Activity                     | Normal          | CGA             | LD              | CGA-LD          |
|------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Fatty acid β-oxidation (nmol of NADH/min/mg of protein) | 22.72 ± 3.37*   | 16.27 ± 1.94*   | 12.70 ± 1.45*   | 22.80 ± 2.50*   |
| CPT-1 (nmol of CoASH/min/mg mitochondrion protein)    | 9.39 ± 0.71*    | 9.55 ± 0.75*    | 6.24 ± 0.64*    | 9.12 ± 0.66*    |
| FAS (nmol of NADPH/min/mg protein)                    | 4.53 ± 0.46*    | 4.60 ± 0.17*    | 6.84 ± 0.43*    | 5.28 ± 0.30*    |

Values are expressed as the mean ± SE, n = 6. The values with different lowercase superscript letters in the same line are significantly different (p<0.05), values with different uppercase superscript letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey’s test.

Fig. 4. CGA up-regulated the expression of p-AMPK in the liver. (A) Western blot analysis of AMPK and p-AMPK expression in the liver. (B) Relative density of p-AMPK. The density of p-AMPK band was normalized to that of AMPK. Values are expressed as the mean ± SE, n = 6. Values with different uppercase letters are significantly different (p<0.01), as analyzed by one-way ANOVA and the Tukey’s test.
Recently, Huang et al. (21) demonstrated 5-caffeoylquinic acid (a kind of CGA) decreased of ACC-α, ACC-β and FAS expression at mRNA levels in high fat diet induced obesity in rat. In our study, the enzymes involved with fatty acid synthesis were decreased in CGA-LD group compared to LD group, such as the content of ACC level was reduced by 43.27%, and the activity of FAS was reduced by 22.81%. Fatty acid β-oxidation was increased by 79.53%, content of CPT-1 (increased by 57.21%), and the CPT-1 activity (increased by 46.15%) in CGA-LD group, as compared with LD group. These results indicated that CGA could decrease fatty acid synthesis and enhance fatty acid oxidation.

In the present study, for the first time to our knowledge, we demonstrated the effect of CGA supplementation on fatty acid composition. The proportion of C18:3n-3 in the liver of CGA-LD rats was significantly increased compared to the LD group. The proportion of C20:1, C24:1, and C18:3n-6 were decreased. C18:3n-3 is a type of ω-3 series fatty acid and C18:3n-6 is a type of ω-6 series fatty acids. The ω-3 series fatty acids have been well studied for their role in reducing the risk factors of disordered lipid metabolism. (28) Supplementation with C18:3n-3 in animals had been shown to decrease hepatic triglyceride concentration (29) and suppress fatty liver formation accompanied with up-regulation of β-oxidation in Zucker fatty rats. (30) C18:3n-6 is a precursor for α-linolenic acid. High concentrations of C18:3n-6 in the overall fatty acid composition is positively correlated with markers of obesity. (31) CGA could up-regulate the level of C18:3n-3 and down-regulate the level of C18:3n-6 in liver, suggesting that this changes may be beneficial on inhibiting fat accumulation in the liver.

In this study, the desaturase activity index of Δ6 and Δ9-18 in CGA-LD group were decreased after CGA supplementation compared to endotoxin infusion rats (LD group). Δ6- and Δ9-desaturases catalyzed desaturation reactions in different fatty acid family metabolic pathways. Δ6-desaturase converted C18:2n-6 to C18:3n-6 and Δ9-desaturase converted C16:0 to C16:1 and C18:0 to C18:1. It has been observed that Δ9-desaturase activity is high in diseases conditions, including diabetes, obesity, and metabolic syndrome. (51) Increased Δ9-desaturase index (18:1/18:0) was found in rectus abdominus muscle of extremely obese subjects with insulin resistance. (52) Warenšjö et al. (53) had been reported that the proportions of C16:0, C18:3n-6. And Δ6-desaturase activity index were significantly correlated with body mass index (BMI) in both women and men.

To further investigate the possible mechanisms of CGA supplementation on fatty acid synthesis and oxidation, the level of AMPK and p-AMPK in liver were measured by western blot. Fatty acid synthesis and oxidation is controlled by AMPK phosphorylation, which stimulates fatty acid β-oxidation and inhibits fatty acid synthesis. From previous studies, the effect of CGA on AMPK has been quite diverse. In vitro, Ong et al. (30) demonstrated that CGA (1–10 mmol/L, 0.5–24 h) stimulated glucose uptake in L6 myoblasts through AMPK. On the other hand, Tsuda et al. (35) reported that no effects of CGA (0.01–1 mmol/L, 5–60 min) treatment on AMPK activation were observed in rat skeletal muscle. The dose and treatment time of CGA may explain the variations in AMPK activation. Recently, chronic administration of CGA (250 mg/kg body weight, 2 weeks) attenuated hepatic steatosis and improved lipid profiles in Lepr db/db mice via AMPK activation. (36) However, Mubarak et al. (37) reported that CGA supplementation in a high fat diet with 1 g/kg diet for 12 weeks did not protect against features of metabolic syndrome in diet-induced obese male C57BL/6 mice, and actually decreased phosphorylation of AMPK. Different strains of animals, the dose of CGA, and experimental timing could alter AMPK activation. In our study, CGA supplementation (60 mg/kg body weight, 4 weeks) significantly increased the level of p-AMPK in the CGA-LD group (p<0.01) compared to the LD group (Fig. 4) and increased fatty acid β-oxidation (Table 4). There was no significant difference in p-AMPK between the normal and CGA groups. In brief, when AMPK was partly inhibited by endotoxin, CGA stimulated AMPK activation.

Metabolic time of CGA after oral administration is considered to be relatively short in Zhou et al. (38) study. They reported that CGA in organs, including liver, was metabolized quickly. And it almost could not be detected in tissues after 4 h of treatment. In our study, CGA treatment significantly increased the level of p-AMPK in the CGA-LD group compared to the LD group in liver sample (which were collected after 12 h fasting). Is such phosphorylation stable for such a long time? Firstly, long-term CGA supplementation altered the level of p-AMPK not only at day 28. As we know, the rats were fed with the chlorogenic acid for 28 days. The level of p-AMPK (phosphorylation) was affected by CGA within 27 days. On the other hand, the metabolites of CGA, such as coffee acid, (25) maybe contribute to the level change of p-AMPK. In further study, we will explore and determine the effect of coffee acid and quinine acid on AMPK in vitro and in vivo.

In conclusion, the present results demonstrated that CGA supplementation ameliorated lipid metabolic disorder in endotoxin-challenged rats. The effect of CGA in modulating lipid metabolism could be attributed to inhibition hepatic fat synthesis and enhancement of fatty acid oxidation, stimulation AMPK activation, as well as modulation fatty acid composition.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (No. 31360552), the Natural Science Foundation of Jiangxi Province (20151BAB204036) and the Open Project Program of State Key Laboratory of Food Science and Technology, Nanchang University (No. SKLF-KF-201414).

Abbreviations

ACC acetyl-CoA carboxylase
ALT alanine transaminase
AMPK AMP-dependent protein kinase
AST aspartate aminotransferase
CGA chlorogenic acid
CPT-1 carnitine palmitoyltransferase
FAS fatty acid synthase
FER food efficiency ratio
HDLC high density lipoprotein cholesterol
LDLC low density lipoprotein cholesterol

References

1 Uner RH, Orci L. Diseases of liporegulation: new perspective on obesity and related disorders. FASEB J 2001; 15: 312–321.
2 Ginsberg IN, Zhang YL, Hernandez-Omo A. Metabolic syndrome: focus on dyslipidemia. Obesity (Silver Spring) 2006; 14 Suppl 1: 418S–498S.
3 van Herpen NA, Schrauwen-Hinderling VB. Lipid accumulation in non-adi- pose tissue and lipotoxicity. Physiol Behav 2008; 94: 231–241.
4 Orellana-Gavaldà JM, Herrero L, Malandrino MI, et al. Molecular therapy for obesity and diabetes based on a long-term increase in hepatic fatty-acid oxidation. Hepatology 2011; 53: 821–832.
5 Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. P Natl Acad Sci USA 1997; 94: 2557–2562.
6 Lassenius MI, Piitiläinen KH, Kaartinen K, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care 2011; 34: 1809–1815.
7 Berg RD. The indigenous gastrointestinal microflora. Trends Microbiol
