

Chlorogenic Acid Improves High Fat Diet-Induced Hepatic Steatosis and Insulin Resistance in Mice

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Received: 17 June 2014 / Accepted: 12 September 2014 / Published online: 24 September 2014
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ABSTRACT

Purpose Chlorogenic acid (CGA), the most abundant component in coffee, has exhibited many biological activities. The objective of this study is to assess preventive and therapeutic effects of CGA on obesity and obesity-related liver steatosis and insulin resistance.

Methods Two sets of experiments were conducted. In set 1, 6-week old C57BL/6 mice were fed a regular chow or high-fat diet (HFD) for 15 weeks with twice intra-peritoneal (IP) injection of CGA (100 mg/kg) or DMSO (carrier solution) per week. In set 2, obese mice (average 50 g) were treated by CGA (100 mg/kg, IP, twice weekly) or DMSO for 6 weeks. Body weight, body composition and food intake were monitored. Blood glucose, insulin and lipid levels were measured at end of the study. Hepatic lipid accumulation and glucose homeostasis were evaluated. Additionally, genes involved in lipid metabolism and inflammation were analyzed by real time PCR.

Results CGA significantly blocked the development of diet-induced obesity but did not affect body weight in obese mice. CGA treatment curbed HFD-induced hepatic steatosis and insulin resistance. Quantitative PCR analysis shows that CGA treatment suppressed hepatic expression of *Ppar γ* , *Cd36*, *Fabp4*, and *Mgat1* gene. CGA treatment also attenuated inflammation in the liver and white adipose tissue accompanied by a decrease in mRNA levels of macrophage marker genes including *F4/80*, *Cd68*, *Cd11b*, *Cd11c*, and *Tnf α* , *Mcp-1* and *Ccr2* encoding inflammatory proteins.

Conclusion Our study provides direct evidence in support of CGA as a potent compound in preventing diet-induced obesity and obesity-related metabolic syndrome. Our results suggest that drinking coffee is beneficial in maintaining metabolic homeostasis when on a high fat diet.

KEY WORDS Chlorogenic acid (CGA) · Hepatic steatosis · Inflammation · Insulin resistance · Obesity

INTRODUCTION

Prevalence of obesity and overweight is progressively expanding worldwide and now affects more than 60% of adults in the US (1,2). Obesity has been identified as the major cause of type 2 diabetes, cardiovascular diseases, various cancers, and other health problems, which lead to further morbidity and mortality. Emergence of metabolic syndrome incidence has resulted in an increased demand for new strategies that are safe and effective in preventing and treating obesity and obesity-associated diseases.

Coffee is one of the most widely consumed beverages, and an epidemiological study has shown coffee is inversely related to the risk of developing chronic diseases, such as type 2 diabetes mellitus, cardiovascular diseases and cancer (3). Among the components in coffee, chlorogenic acid (CGA), an ester of caffeic acid and quinic acid (4), is the most abundant form of phenolic acid and major bioactive compound, which is also widely available in other plants, fruits and vegetables such as apples, pears, tomatoes, blueberries, and many others (5). In addition to its strong antioxidative property, the potential role of CGA in glucose and lipid metabolism has been explored in recent years. CGA has been reported to inhibit glucose-6-phosphate translocase 1, resulting in the reduction of glucose transport in the intestine (6). In the meanwhile, CGA enhances glucose uptake in isolated skeletal muscle cells and L6 muscle cell line (7,8) and improves glucose tolerance in *db/db* mice (9), indicating its potential antidiabetic activity. In addition, CGA significantly decreased plasma and liver lipid levels in (*fa/fa*) Zucker rats and rats fed a high-cholesterol diet (10,11). Using a fat enriched diet containing 37% fat calorie and 0.02% of CGA, Cho and colleagues (12) showed a reduced weight gain of ICR mice by 16% (40.05 g vs

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47.7 g for high fat diet control without CGA). These studies suggest that CGA may exert a beneficial influence on metabolic diseases. The current study investigates the preventive and therapeutic activity of CGA in mice fed a HFD. Special attention was paid to the CGA effect on obesity-related liver steatosis and insulin resistance and the underlying molecular mechanism. Our results demonstrate that CGA significantly blocked diet-induced weight gain but did not affect body weight or fat mass in obese mice. In both prevention and treatment studies, CGA suppressed hepatic steatosis, suppressed obesity-related inflammation and improved glucose tolerance and insulin sensitivity. Our results provide critical information regarding the beneficial effects of CGA in managing obesity and obesity-associated metabolic disorders.

MATERIALS AND METHODS

Materials

Chlorogenic acid (CGA) was purchased from Cayman Chemical (Ann Arbor, Michigan). The TRIZOL reagent and the SuperScript® III First-Strand Synthesis System are from Life Technologies (Grand Island, NY). The RNeasy Lipid Tissue Mini Kit was from Qiagen (Valencia, CA). PerfeCTa® SYBR® Green FastMix was acquired from Quanta BioSciences (Gaithersburg, MD). The Oil Red O solution was obtained from Electron Microscopy Science (Hatfield, PA). Infinity™ Triglycerides kit was purchased from Fisher Diagnostics (Middletown, VA). Total cholesterol assay kit was from Genzyme Diagnostics (Charlottetown, PE Canada) and NEFA-HR assay kits for free fatty acid was from Wako Bioproducts (Richmond, VA). The Mercodia Insulin ELISA kit was purchased from Mercodia Developing Diagnostics (Winston Salem, NC). A TUREtrack glucometer and test strips were purchased from Nipro Diagnostics, Inc. (Fort Lauderdale, FL). High-fat diet (F3282, 60% kJ/fat) was purchased from Bio-serv (Frenchtown, NJ). C57BL/6 mice were purchased from Charles River (Wilmington, MA).

Animals and Treatment

All procedures performed on mice were approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, Georgia. Two sets of experiments were carried out. In the first set, 6-week-old male C57BL/6 mice fed a HFD received two injections of CGA (100 mg/kg, intra-peritoneal) per week or carrier solution [dimethyl sulfoxide (DMSO)] for 15 weeks. In the second, obese mice (average body weight 50 g) were administrated CGA (100 mg/kg, intra-peritoneal) or DMSO twice per week for 6 weeks. Body weight and food intake were monitored weekly

and animal body composition was determined at the end of the experiment using EchoMRI-100™ from Echo Medical Systems (Houston, TX).

Histochemical Analysis

After mice were sacrificed, the liver and epididymal white adipose tissue (eWAT) and brown adipose tissues (BAT) were collected, fixed in 10% formalin, embedded in paraffin, and sectioned at a thickness of 6 µm. Hematoxylin and eosin (H&E) staining was performed. Frozen sections (8 µm) were stained with 0.2% Oil Red O in 60% of isopropanol for 15 min and washed three times with phosphate buffered saline. A microscopic examination was performed and photographs were taken under a regular light microscope.

Lipid Extraction and Analysis

Hepatic lipids were extracted following an established procedure (13). Briefly, liver tissues were homogenized in phosphate buffered saline. Total lipids in 300 µl of homogenate were extracted by addition of 5 ml of chloroform-methanol (2:1, vol/vol) mixture. An aliquot of the organic phase was evaporated to dry and dissolved in 1% Triton X-100. Hepatic cholesterol and triglyceride assays were performed according to the manufacturer's instructions.

Determination of Blood Lipid and Insulin Level

Blood samples were collected from fasted mice. Cholesterol, triglyceride, free fatty acid and insulin levels in the plasma were measured using commercial assay kits according to the manufacturer's instructions.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

For GTT, mice were injected intraperitoneally with glucose at 2 g/kg body weight after fasting overnight. A small cut at the tip of a mouse tail was made at a selected time to give a small drop of blood which was directed absorbed into a test strip for determination of glucose level using a glucometer. For ITT, mice fasted for 4 h and blood glucose levels were measured after an intraperitoneal injection of insulin (0.75 U/kg) from Eli Lilly (Indianapolis, IN).

Gene Expression Analysis by Real Time PCR

Total RNA was isolated from the mouse liver, pancreas, and white adipose tissues using the TRIZOL reagent or an RNeasy kit. Two micrograms of total RNA were used for the first strand cDNA synthesis, as recommended by the manufacturer. Real time PCR was performed using SYBR

Green as an indicator using ABI StepOne Plus Real Time PCR system. PCR was carried out for 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. The data were normalized using GAPDH mRNA as an internal control. The primer sequences employed are summarized in Table 1.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). One-way ANOVA was performed to evaluate the difference following Tukey post hoc test. All data are reported as mean \pm standard deviation (SD) with statistical significance set at $p < 0.05$.

RESULTS

CGA Prevented Animals from Development of High-Fat Diet-Induced Obesity and Macrophage Infiltration in eWAT

In the first set of experiments, the protective activity of CGA in diet-induced obesity was examined. As shown in Fig. 1a, 15-week HFD feeding significantly induced weight gain, and the average body weight in control animals is 48.2 ± 2.4 g. The average body weight of CGA-treated animals, on the other hand, is 31.6 ± 2.0 g at the end of the 15-week HFD feeding, slightly above the average body weight of chow-fed mice, but about 16 g less than that of HFD-fed control. Results from body composition analysis show that CGA blocked the increase of fat mass in HFD-fed animals without affecting lean mass (Fig. 1b). Compared to HFD-fed control animals, the CGA injection did not affect food intake during the 15-week feeding period (Fig. 1c). H&E staining of eWAT and BAT confirms lower level of fat accumulation in adipocytes in CGA treated mice, judging by the average size of adipocytes in eWAT (Fig. 1d–g) and the density of vacuole type structure in BAT (Fig. 1h–j). These results demonstrate that CGA blocks lipid accumulation in adipose tissue.

Macrophage infiltration in WAT is commonly observed in HFD-induced obesity (12,13). We examined the levels of adipose macrophages in control and CGA-treated mice using RT-PCR analysis. Figure 2 shows that HFD increased mRNA levels for macrophage specific marker genes *F4/80*, *Cd68*, *Cd11c*, and *Cd11b* by 9.2-, 20.2-, 9.6-, and 33.3-fold, respectively, compared to control animals fed regular chow. The transcript levels in the same group of macrophage marker genes in CGA-treated animals are significantly less than the HFD-fed control and similar to that of animals fed regular chow. These results suggest that CGA blocks HFD-

induced chronic inflammation and macrophage infiltration in WAT.

CGA Suppressed PPAR γ -Associated Development of Fatty Liver in HFD-fed Mice

In addition to increased fat tissue, hepatic steatosis is also commonly observed in diet-induced obesity (14). Results in Fig. 3a–f show that HFD induced significant lipid accumulation in the mouse liver, as evidenced by the extensive vacuolation in H&E stained liver section and Oil Red O-stained massive lipid droplets. However, CGA-treated animals displayed significantly less fat accumulation in the liver, to levels similar to mice fed regular chow, which was also confirmed by measuring extracted liver triglyceride levels (Fig. 3g). The effects of CGA on blood concentration of triglyceride, free fatty acid and cholesterol were also examined. As shown in Fig. 3h–j, CGA significantly lowered triglyceride and cholesterol level but did not affect blood concentration of free fatty acid. Taken together, these results demonstrate that CGA markedly reduces hepatic lipid levels and improves plasma lipid profile.

Further studies were performed to evaluate the effect of CGA treatment on expression of genes involved in hepatic fat accumulation. Figure 4a shows that CGA markedly suppressed HFD-enhanced mRNA levels of peroxisome proliferator-activated receptor gamma (*Ppar γ 1*) (63%), *Ppar γ 2* (52%), and its target genes including *Cd36* (83%), fatty acid binding protein 4 (*Fabp4*, 47%), and monoacylglycerol O-acyltransferase 1 (*Mgat1*, 99%). In addition, CGA treatment also increased mRNA levels of genes involving fatty acid metabolism including carnitine palmitoyltransferase I (*Cpt1a*, *Cpt1b*), and fibroblast growth factor 21 (*Fgf21*) (Fig. 4b). CGA also inhibited HFD-induced hepatic inflammation, as evidenced by suppression of *Cd68*, *Cd11b* and *Cd11c* gene expression (Fig. 4c).

CGA Suppressed Diet-Induced Hyperinsulinemia and Hyperglycemia in HFD-Fed Mice

To test whether CGA treatment improves obesity-associated insulin resistance, we compared fasting glucose levels and degree of glucose intolerance in the CGA-treated and DMSO-injected control mice fed a HFD. Levels of fasting glucose were much lower in CGA-treated mice than the HFD-fed control (134 ± 28 mg/dl *vs.* 204 ± 43 mg/dl). A glucose tolerance test (Fig. 5a) and the calculated area under the curve (AUC) (Fig. 5b) revealed a much higher clearance rate of intra-peritoneally injected glucose in CGA-treated animals, suggesting that CGA maintains glucose sensitivity. Protection against diet-induced insulin resistance was also demonstrated by an insulin tolerance test (Fig. 5c). Accordingly, CGA also ameliorated diet-induced hyperinsulinemia ($0.7 \mu\text{g/l}$ *vs.*

Table 1 Primer Sets for Real Time PCR Analysis of Gene Expression

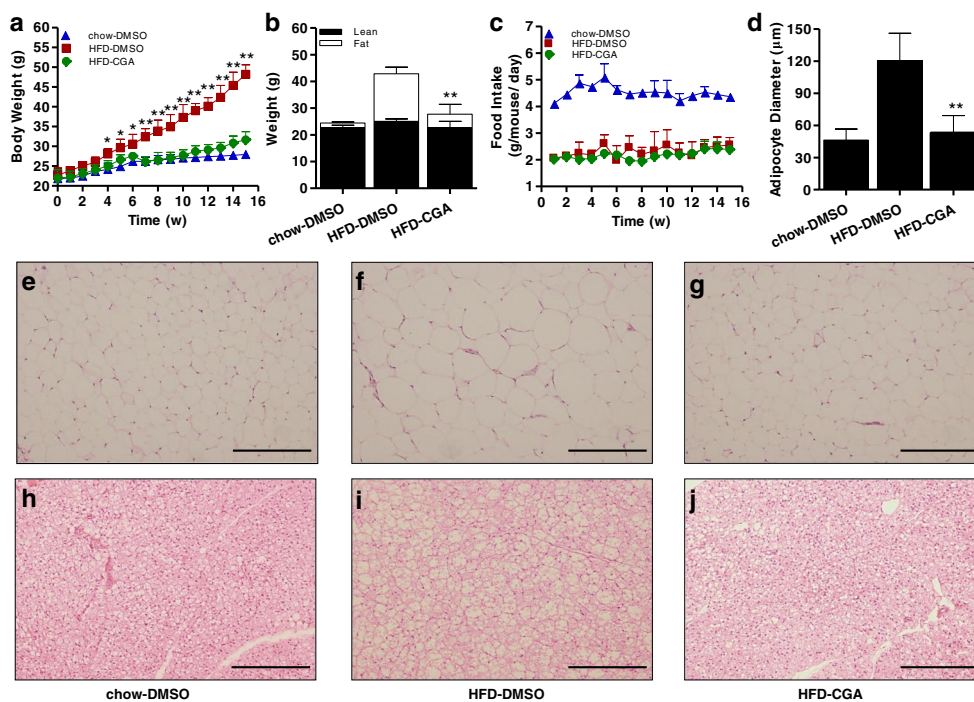
Name	Forward sequence	Reverse sequence
<i>F4/80</i>	CCCCAGTGTCTTACAGAGTG	GTGCCAGAGTGGATGTCT
<i>Cd68</i>	CCATCCTTCACGATGACACCT	GGCAGGGTTATGAGTGACAGTT
<i>Cd11b</i>	ATGGACGCTGATGGCAATACC	TCCCCATTACAGTCTCCCA
<i>Cd11c</i>	ACGTCAGTACAAGGAGATGTTGGA	ATCCTATTGCAGAATGCTTCTTTACC
<i>Mcp-1</i>	ACTGAAGCCAGCTCTCTCTTCCTC	TTCTTCTTGGGGTCAGCACAGAC
<i>Tnfa</i>	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
<i>Pparγ1</i>	GGAAGACCACTCGCATTCTCTT	GTAATCAGCAACCATTGGGTCA
<i>Pparγ2</i>	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
<i>Cd36</i>	CCTTAAAGGAATCCCCGTGT	TGCATTTGCCAATGTCTAGC
<i>Fabp4</i>	AAGGTGAAGAGCATCATAACCC	TCACGCCTTTTATAACACATTCC
<i>Mgat1</i>	TGGTGCCAGTTTGGTCCAG	TGCTCTGAGGTCCGGTTCA
<i>Cpt1a</i>	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
<i>Cpt1b</i>	GGTCTCTTCTCAAGGTCTG	CGAGGATTCTCTGGAAGTGC
<i>Fgf21</i>	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCAGTGTTC
<i>Insulin1</i>	CACTTCCTACCCCTGCTGG	ACCACAAAGATGCTGTTTGACA
<i>Insulin2</i>	GCTTCTTCTACACACCCATGTC	AGCACTGATCTACAATGCCAC
<i>Ppara</i>	TGTCGAATATGTGGGGACAA	AATCTTGAGCTCCGATCAC
<i>Acox1</i>	CCGCAACCTTCAATCCAGAG	CAAGTTCTCGATTCTCGACGG
<i>Ccr2</i>	ATCCACGGCATACTATCAACATC	CAAGGCTCACCATCATCGTAG
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

5.3 μg/l) as shown in Fig. 5d, and dramatically inhibited an HFD-induced increase in mRNA levels of pancreatic *Insulin1* and *Insulin2* genes (Fig. 5e, f). These results validate that CGA protects animals against obesity-induced insulin resistance.

CGA Treatment did not Affect Body Weight but Improved Insulin Sensitivity in Obese Mice

While the preventive effect of CGA on HFD-induced obesity, fatty liver and insulin resistance is clear, we question whether

Fig. 1 CGA prevented HFD-induced obesity in C57BL/6 male mice. Six-week-old C57BL/6 male mice were fed regular chow or HFD for 15 weeks. Mice were intraperitoneally injected by CGA (100 mg/kg, twice weekly) or carrier solution (DMSO) as the control. **(a)** Growth curve. **(b)** Fat and lean mass. **(c)** Food intake. **(d)** Average diameter of adipocytes in epididymal fat pad (calculated from 200 adipocytes from 4 separate slides). **(e–g)** H&E staining of white adipose tissue, scale bar: 500 μm. **(h–j)** H&E staining of brown adipose tissue, scale bar: 500 μm. Each data point represents the Mean ± SD of 5 animals. * $P < 0.05$, ** $P < 0.01$ compared to HFD-DMSO group.



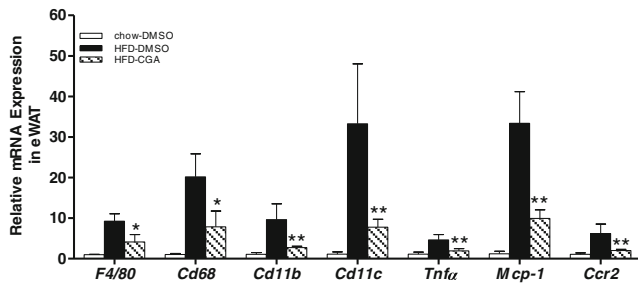


Fig. 2 Reduction of HFD-induced inflammation in epididymal white adipose tissue by CGA administration. Mice were sacrificed after 15-week of CGA injection. Epididymal adipose tissues were harvested, and total RNA was extracted. Relative mRNA levels of selected genes were determined by real time PCR. Inflammation is represented by the following selected gene expression: *F4/80*, *Cd68*, *Cd11b*, and *Cd11c* as well as cytokine *Tnfa*, and chemotactic factor gene *Mcp-1* and its receptor *Ccr2* in eWAT. Each data point represents the Mean \pm SD, ($n = 4$). * $P < 0.05$, ** $P < 0.01$ compared to HFD-DMSO group.

CGA has a therapeutic effect on obese mice. Diet-induced obese mice (average body weight 50 g) were divided into two groups and treated intraperitoneally with CGA (100 mg/kg), or carrier solution (DMSO) twice weekly for 6 weeks. Results in Fig. 6a–c show that 6-week CGA treatment did not induce weight loss and the body fat mass and lean mass remained the same between CGA treated and control animals. H&E staining of eWAT shows that CGA treatment did not significantly change the size of adipocytes in WAT but slightly reduced the lipid droplets in BAT (Fig. 6d–g). Glucose tolerance and insulin sensitivity tests reveal that CGA treatment improved insulin

sensitivity in obese mice, which is confirmed by amelioration of obesity-related hyperinsulinemia and reduction of calculated HOMA-IR. Taken together, these data demonstrate that CGA treatment improves insulin sensitivity in obese mice.

CGA Treatment Reduced Hepatic Lipid Accumulation and Obesity-Related Chronic Inflammation in Obese Mice

Although CGA treatment did not induce weight loss in obese mice, results in Fig. 7e demonstrate that liver weight in CGA-treated animal was ~ 1.5 g, significantly lower than that of controls at ~ 2.5 g. In line with reduced liver weight, H&E and Oil red O staining of liver sections show less lipid accumulation in the liver of CGA-treated mice (Fig. 7a–d). Consistent with these results, CGA treatment of mice significantly reduced hepatic total cholesterol and triglyceride levels in obese animals (Fig. 7f, g). CGA treatment also reduced blood concentrations of free fatty acid, cholesterol and triglyceride levels (Fig. 7h–j).

Similar to prevention study, mRNA levels of critical set of genes involved in lipid metabolism and inflammation were determined. Results in Fig. 8a show that CGA treatment decreased mRNA levels of *Ppar γ 1* and *Ppar γ 2*, and their target genes *Cd36*, *Fabp4* and *Mgat1*. In addition, CGA treatment also enhanced the *Ppara* level by 45% and increased its target genes peroxisomal acyl-coenzyme A oxidase 1 (*Acox1*) and *Fgf21* by 3.3~ and 1.8~ fold, respectively. *Cpt1* gene transcription was not affected by CGA treatment (Fig. 8b).

Fig. 3 CGA repressed HFD-induced hepatic steatosis and serum lipid levels. At the end of the 15-week of feeding, mice were sacrificed and livers were fixed with 10% neutrally buffered formalin. (a–c) Images of liver sections with H&E staining, scale bar: 500 μ m. (d–f) Lipid level revealed by Oil Red O staining, scale bar: 500 μ m. (g) Hepatic triglyceride levels. (h–j) Serum levels of triglyceride, total cholesterol and free fatty acid. * $P < 0.05$ compared to HFD-DMSO group.

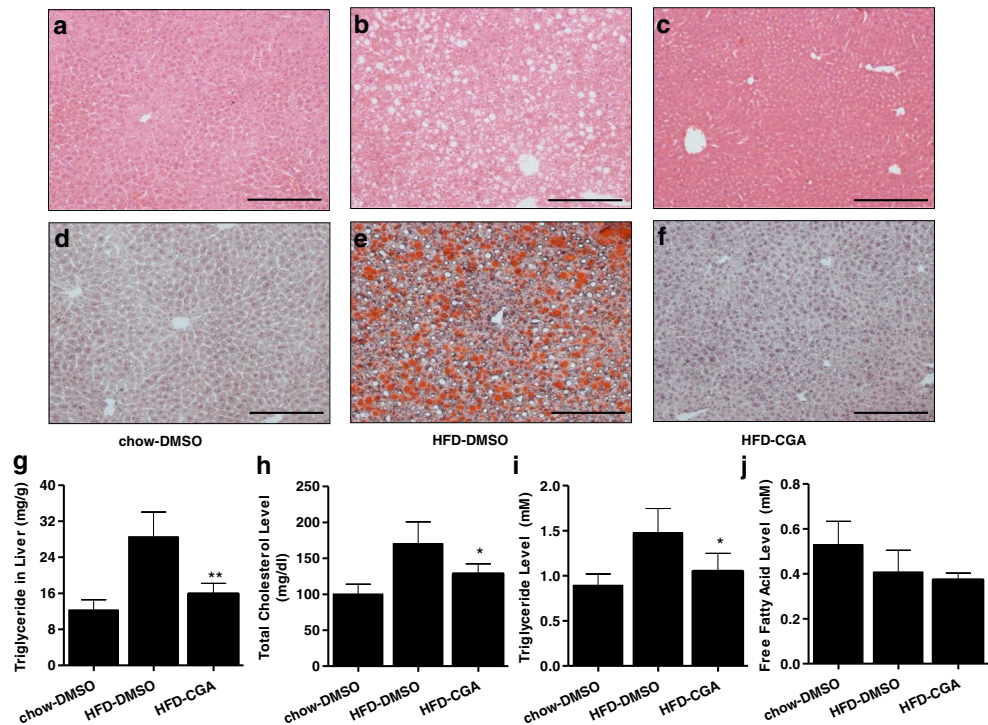
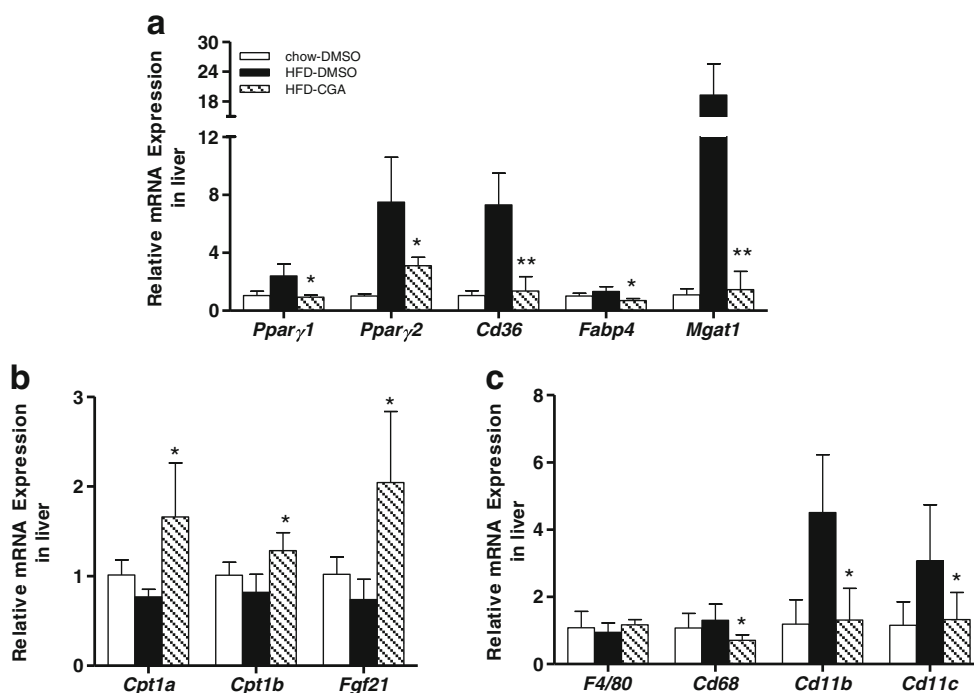


Fig. 4 Effects of CGA on hepatic gene expression involved in lipid metabolism and inflammation. After mice were sacrificed at the end of 15-week of HFD feeding, liver tissues were harvested and hepatic total RNA was extracted. Real-time PCR analysis was utilized to evaluate relative mRNA level of selected genes which included (a) *Ppar γ 1*, *Ppar γ 2* and target genes *Cd36*, *Fabp4* and *Mgat1*. (b) *Cpt1a*, *Cpt1b* and *Fgf21*. (c) Macrophage marker genes *F4/80*, *Cd68*, *Cd11b*, and *Cd11c*. Each data point represents the Mean \pm SD, ($n=4$). * $P<0.05$, ** $P<0.01$ compared to HFD-DMSO group.

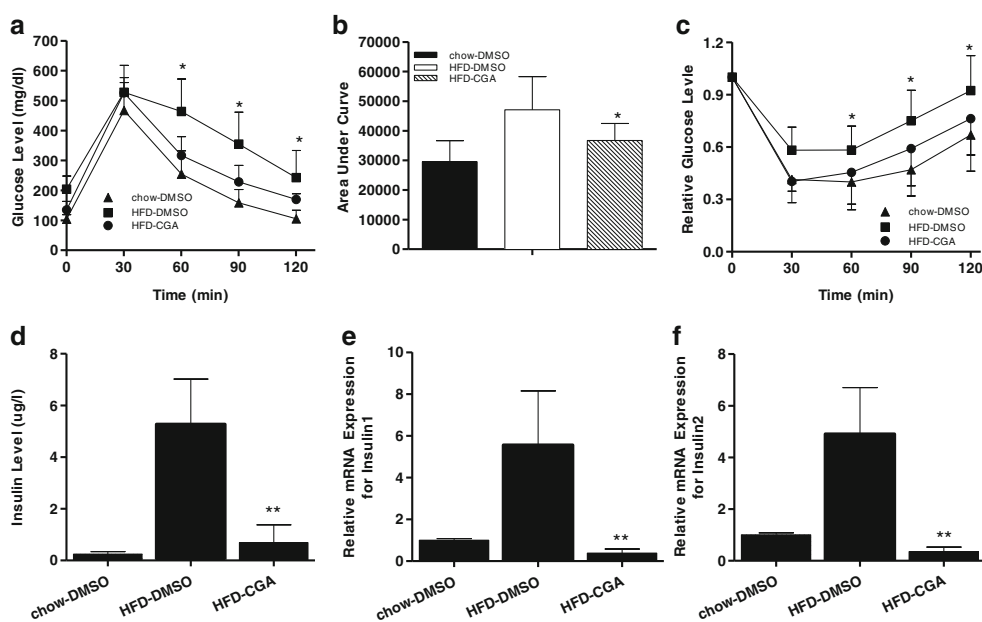


Consistent with improved hepatic lipid level, expression of macrophage marker genes such as chemoattractant *Mcp-1* and its receptor C-C chemokine receptor type 2 (*Ccr2*), *F4/80*, *Cd68* as well as *Tnfa* were also lower in CGA-treated animals (Fig. 8c). Similarly, mRNA levels of the same set of genes in eWAT also significantly reduced by CGA treatment (Fig. 8d). Taken together, these data suggest that CGA treatment of obese mice reduces HFD-induced chronic inflammation and improved hepatic steatosis.

DISCUSSION

Previous studies have demonstrated that CGA is capable of effecting glucose and lipid metabolism as well as weight gain induced by HFD feeding (6,9–12). The current study comprehensively assesses both preventive and therapeutic activities of CGA on diet-induced obesity and obesity-associated metabolic syndromes, demonstrates the effect of CGA on obesity-related liver steatosis and insulin resistance, and explores underlying molecular mechanism. Utilizing C57BL/6 mice fed a HFD

Fig. 5 CGA improved hyperinsulinemia and hyperglycemia of animals fed with HFD. (a) Time dependent blood glucose level followed by IP injection of glucose (2 g/kg). (b) Area under the curve from glucose tolerance test. (c) Time dependent ratio of glucose concentration upon IP injection of insulin (0.75 U/kg). (d) Serum insulin levels at the end of the 15-week feeding with or without CGA administration. (e, f) Relative mRNA levels of *Insulin1* and *Insulin2* in pancreas. Each data point represents the Mean \pm SD of 5 animals. * $P<0.05$, ** $P<0.01$ compared to HFD-DMSO group.



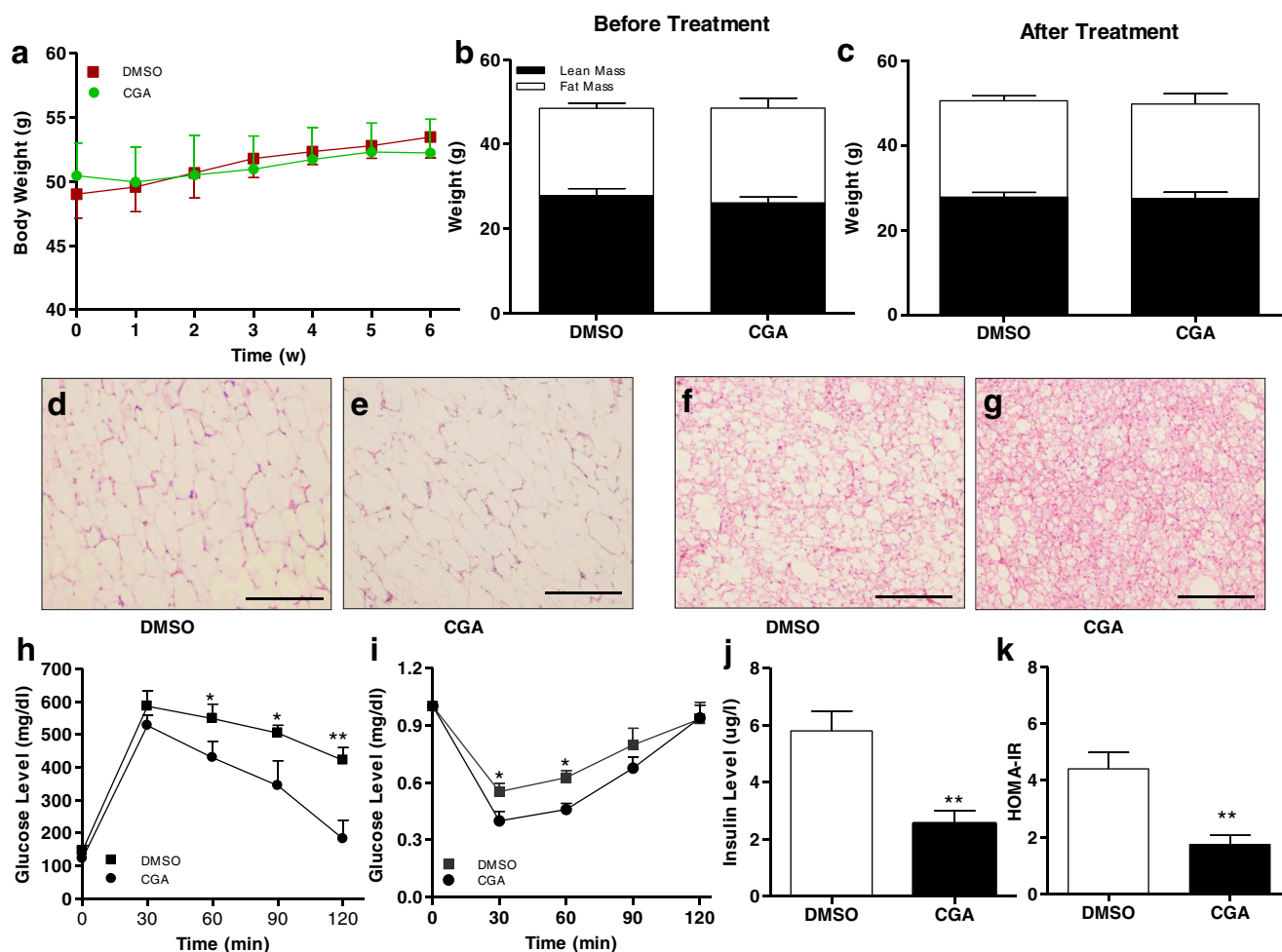


Fig. 6 Six-week CGA treatment failed to cause weight loss in obese mice but improved insulin sensitivity. C57BL/6 obese mice fed a HFD were treated with CGA (100 mg/kg) or DMSO for 6 week. **(a)** Change of body weight. **(b, c)** Fat and lean mass measured before and after 6-week treatment. **(d, e)** H&E staining of white adipose tissue; scale bar: 500 μ m. **(f, g)** H&E staining of brown adipose tissue; scale bar: 500 μ m. **(h)** Time-dependent blood glucose level followed by IP injection of glucose (2 g/kg). **(i)** Time-dependent ratio of glucose concentration upon IP injection of insulin (0.75U/kg). **(j)** Serum insulin levels at the end of the 6-week CGA treatment; **(k)** HOMA-IR values calculated based on formula: (fasting insulin [μ U/ml] \times fasting glucose [mmol/l])/22.5. Each data point represents the Mean \pm SD of 5 animals. * $P < 0.05$, ** $P < 0.01$ compared to DMSO group.

with 60% calorie from fats as an animal model and intraperitoneal injection as a route of drug administration, we examined the CGA activity in blocking HFD-induced weight gain and in reducing the body weight of obese mice. We demonstrate that CGA was effective in preventing HFD-induced weight gain (Fig. 1), inhibiting development of liver steatosis (Fig. 3), and blocking HFD-induced insulin resistance (Fig. 5). CGA treatment of obese mice did not yield weight loss, but improved insulin sensitivity (Fig. 6) and reduced lipid accumulation in the liver (Fig. 7). The beneficial effects were associated with CGA activity in blocking HFD-induced inflammation (Fig. 2), inhibiting diet-induced expression of *Ppar γ* and its target genes in the liver, and increasing expression of genes responsible for lipid metabolism (Figs. 4 and 8).

The protective activity of CGA against HFD-induced obesity can be attributed to its antioxidant activity. Accumulating evidence suggests that chronic inflammation is closely associated with diet-induced obesity (15). HFD elevates production of

reactive oxygen species in adipose tissue and boosts macrophage infiltration (16,17). We have previously shown that macrophage elimination by clodronate liposomes blocks HFD-induced obesity (18). We also recently demonstrated that HFD-induced obesity could be blocked by overexpression of superoxide dismutase 3 gene as a means to maintain redox homeostasis (19). CGA is a well-known anti-oxidant and capable of suppressing inflammation by inhibiting NF- κ B and JNK/AP-1 activation and inhibition of the toll-like receptor 4 signaling pathway (20,21). Consistent with these results, we observed that CGA treatment greatly reduced the mRNA level of macrophage marker genes in eWAT including *F4/80*, *Cd68*, *Cd11b*, *Cd11c*, *Tnfa* and *Mcp-1* (Fig. 2), indicating a lack of diet-induced macrophage infiltration. Mechanistically, twice injection of CGA into mice per week provides sufficient antioxidants for animals to remove reactive oxygen species generated by consumption of lipids-enriched diet, thereby suppressing the expression of the pro-inflammatory cytokine gene induced by

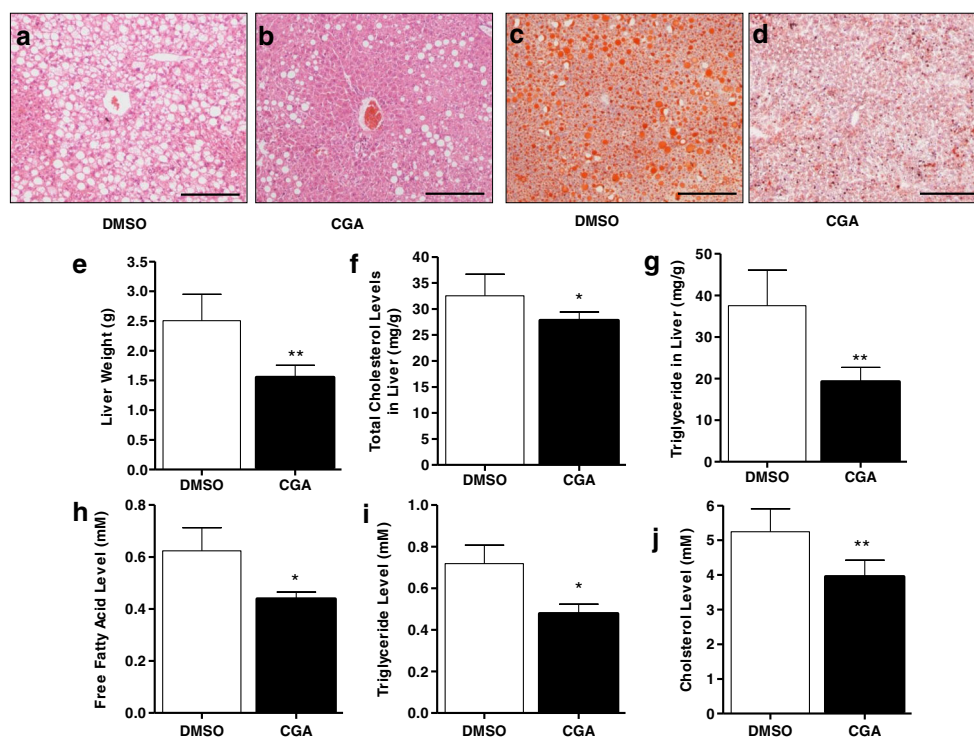


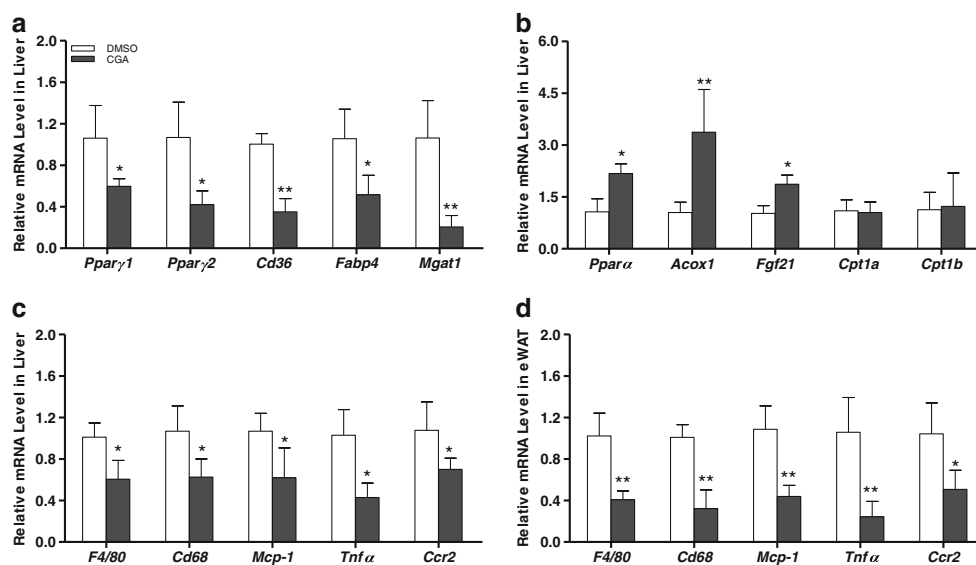
Fig. 7 CGA treatment reduced hepatic and blood lipids levels in obese mice fed a HFD. (a, b) Liver sections stained with H&E. (c, d) Oil Red O staining of liver sections showing less lipid accumulation in livers of CGA-treated animals compared with that of control. (e) Liver weight at the end of the 6-week treatment in obese mice. (f, g) Hepatic total cholesterol and triglyceride. (h-j) Serum levels of free fatty acid, cholesterol and triglyceride. Each data point represents the Mean \pm SD ($n=5$). * $P < 0.05$, ** $P < 0.01$ compared to DMSO-treated groups.

HFD, and consequently blocking fat accumulation and weight gain. Once the development of obesity is blocked, other metabolic parameters such glucose and insulin sensitivity maintain normal (Fig. 5). Results shown in Fig. 6a suggest that blockade of inflammation is not sufficient in reducing body weight in fats already accumulated in adipose tissue, indicating that antioxidants would not be effective in reducing body weight.

Results in Figs. 4 and 8 clearly show that CGA prevented and improved liver steatosis by inhibiting the PPAR γ

pathway. PPAR γ , especially PPAR γ 2, is expressed highly in adipose tissue (22,23), promoting fatty acid uptake into adipocytes and adipocyte differentiation. Overexpression of hepatic *Ppar γ* resulted in exacerbated liver steatosis (24). Conversely, liver and hepatocyte specific *Ppar γ* knockout mice were protected against hepatic lipid accumulation (25–27). These studies suggest that PPAR γ plays an important role in the development of hepatic steatosis. Our results show that HFD stimulated both *Ppar γ 1* and *Ppar γ 2* gene expression

Fig. 8 Effect of CGA treatment on gene expressions in liver and white adipose tissue which were involved in lipid metabolism and inflammation. At the end of the 6-week CGA treatment of obese mice, animals were sacrificed and total RNA extracted from liver and white adipose tissue. (a) Relative mRNA levels of *Ppar γ 1*, *Ppar γ 2* and their target genes *Cd36*, *Fabp4* and *Mgat1*. (b) mRNA levels of *Ppar α* , *Acox1*, *Fgf21*, *Cpt1a* and *Cpt1b*. (c, d) mRNA levels of macrophage gene markers *F4/80*, *Cd68* and inflammatory factor genes *Tnf α* , *Mcp-1* and *Ccr2*. Each data point represents the Mean \pm SD, ($n=4$). * $P < 0.05$, ** $P < 0.01$ compared to DMSO group.



in the liver, concordant with HFD-induced hepatic lipid accumulation. Hepatic fatty acid transporter CD36 is a common target of PPAR γ (28) and increased hepatic CD36 activity is critical for the development of steatosis in obesity (29–31). Consistent with previous reports (13,32–34), HFD significantly induced hepatic *Cd36* expression, facilitating transport of long-chain fatty acids into liver. In both prevention and treatment studies, CGA dramatically reduced hepatic *Ppar γ* mRNA level and inhibited expression of fatty acid transporter *Cd36* and *Fabp4* genes (Figs. 4a and 8a). In addition to blockade of fatty acid uptake, CGA also dramatically inhibited PPAR γ -regulated *Mgat1* expression. *Mgat1* is involved in incorporation of fatty acids into triglyceride (35) and knockdown of this enzyme in the liver markedly reduced hepatic steatosis in diet-induced and genetic obese model (36). Therefore, by targeting the hepatic PPAR γ pathway, CGA exerts its protective function against obesity-induced hepatic steatosis. Here, we quantify the change of PPAR γ and its target genes by real time PCR. It should be noticed that the gene expression level is not necessarily equal to the protein level. Measurement in protein level will further confirm the effect of CGA on this pathway. In addition to the effect on Ppar γ , previous studies have shown that CGA could enhance PPAR α level in the liver and stimulate lipid utilization (11,12). In consistent with these earlier reports, our data also show that CGA promoted expression of *Ppar α* and its target genes *Acox1* and *Fgf21* (Figs. 4b and 8b).

Several lines of evidence suggest CGA is a novel insulin sensitizer and capable of maintaining glucose hemostasis. Daily consumption of more than 3 cups of decaffeinated coffee containing high contents of CGA lowers the risk of developing type-2 diabetes by 30% (37). Clinical trials also verify that EmulinTM, a patented blend of chlorogenic acid,

myricetin, and quercetin, is able to chronically lower background blood glucose levels of diabetic patients (38). Here, our data demonstrate that CGA prevented HFD-induced hyperglycemia and hyperinsulinemia (Fig. 5). Accordingly, CGA also improved insulin sensitivity in obese mice (Fig. 6h–k), in agreement with previous reports in *db/db* mice (9) and streptozotocin (STZ)-induced diabetic rats (39). Recent studies suggest that the inflamed adipose tissue in obesity triggers the development of obesity-related insulin resistance (40), indicating that reduction of tissue inflammation may be beneficial in obesity-related metabolic syndromes. In line with this report, RT-PCR analysis demonstrates that CGA inhibited diet-induced expression of macrophage marker genes (*F4/80*, *Cd68*, *Cd11b*, and *Cd11c*) and inhibited expression of pro-inflammatory mediator genes (*Tnf α* and *Mcp-1*) in the macrophages (Figs. 2 and 8). Attenuation of inflammatory phenotypes in adipose tissue and liver by CGA sensitizes peripheral tissues for the insulin response. In addition, Ong *et al.* (8) reported recently that CGA enables activation of AMPK and increase of glucose transport in skeletal muscle isolated from *db/db* mice and L6 skeletal muscle cells. In the meantime, CGA is capable of inhibiting hepatic gluconeogenesis by suppressing G6Pase activity (9). These potential effects may also contribute to the influence of CGA in obesity-related insulin resistance. The pathways for CGA-mediated beneficial effects against HFD-induced obesity are illustrated in Fig. 9.

In summary, results presented in this study demonstrate CGA prevents mice from diet-induced obesity and obesity-related metabolic syndrome. CGA also improves liver steatosis, insulin sensitivity and reduces chronic inflammation in obese mice. These findings provide direct evidence in support of the potential health benefits of CGA in managing obesity and obesity-associated metabolic disorders. The next step for CGA research may be the development of improved CGA formulation to reach an effective CGA concentration in humans without toxicity and inconvenience.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported in part by the National Institute of Health (RO1EB007357 and RO1HL098295). We thank Ms. Ryan Fugett for proof-reading and English editing.

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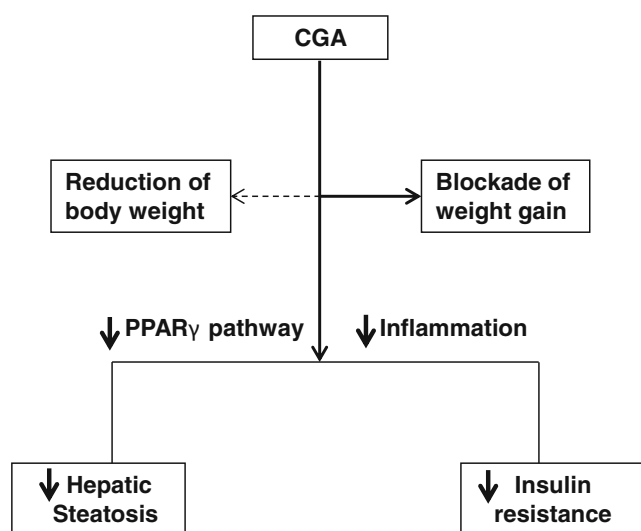


Fig. 9 Summary of CGA-mediated beneficial effects in high fat diet induced obesity and obesity-associated liver steatosis and insulin resistance. Solid arrow indicates positive effect. Dash arrow indicates no effect.

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