



Chlorogenic acid and caffeine in combination inhibit fat accumulation by regulating hepatic lipid metabolism-related enzymes in mice

Guodong Zheng*, Yangyang Qiu, Qing-Feng Zhang and Dongming Li

Jiangxi Key Laboratory of Natural Product and Functional Food, College of Food Science and Engineering, Jiangxi Agricultural University, Nanchang 330045, People's Republic of China

(Submitted 29 July 2013 – Final revision received 17 November 2013 – Accepted 21 February 2014 – First published online 24 July 2014)

Abstract

Obesity has become a public health concern due to its positive association with the incidence of many diseases, and coffee components including chlorogenic acid (CGA) and caffeine have been demonstrated to play roles in the suppression of fat accumulation. To investigate the mechanism by which CGA and caffeine regulate lipid metabolism, in the present study, forty mice were randomly assigned to four groups and fed diets containing no CGA or caffeine, CGA, caffeine, or CGA + caffeine for 24 weeks. Body weight, intraperitoneal adipose tissue (IPAT) weight, and serum biochemical parameters were measured, and the activities and mRNA and protein expression of lipid metabolism-related enzymes were analysed. There was a decrease in the body weight and IPAT weight of mice fed the CGA + caffeine diet. There was a significant decrease in the serum and hepatic concentrations of total cholesterol, TAG and leptin of mice fed the CGA + caffeine diet. The activities of carnitine acyltransferase (CAT) and acyl-CoA oxidase (ACO) were increased in mice fed the caffeine and CGA + caffeine diets, while the activity of fatty acid synthase (FAS) was suppressed in those fed the CGA + caffeine diet. The mRNA expression levels of AMP-activated protein kinase (AMPK), CAT and ACO were considerably up-regulated in mice fed the CGA + caffeine diet, while those of PPAR γ 2 were down-regulated. The protein expression levels of AMPK were increased and those of FAS were decreased in mice fed the CGA + caffeine diet. These results indicate that CGA + caffeine suppresses fat accumulation and body weight gain by regulating the activities and mRNA and protein expression levels of hepatic lipid metabolism-related enzymes and that these effects are stronger than those exerted by CGA and caffeine individually.

Key words: Chlorogenic acid: Caffeine: Fat accumulation: Lipid metabolism-related enzymes

Obesity has become a public health concern due to its positive association with the incidence of CVD, type 2 diabetes mellitus, hypertension, stroke, dyslipidaemia, osteoarthritis and some cancers⁽¹⁾. Therefore, prevention of obesity is a prophylactic measure for many of these diseases.

Coffee beans contain several bioactive components such as chlorogenic acid (CGA) and caffeine. CGA, the ester of caffeic acid and quinic acid, is one of the most abundant polyphenols in the human diet and has been reported to decrease the incidence of chemical carcinogenesis in several animal models of cancer and to suppress the growth of bacteria⁽²⁾. CGA has various biological properties, such as antioxidant, anti-mutagenic, anti-cancer and matrix metalloproteinase-inhibiting properties⁽³⁾. CGA has been claimed not only to delay glucose absorption in the intestine but also to decrease hepatic glucose output through the inhibition of glucose-6-phosphatase activity⁽⁴⁾. Cho *et al.*⁽⁵⁾ have demonstrated that CGA significantly lowers the concentrations of NEFA, TAG

and total cholesterol (TC) in the serum. It has also been reported that 0.1% CGA significantly enhances the BMR of rats fed a high-fat diet⁽⁶⁾. Moreover, Hsu *et al.*⁽⁷⁾ have reported that CGA inhibits the growth of preadipocyte populations. Caffeine ingestion reduces the size of adipose pads and the number of adipocytes⁽⁸⁾ and enhances the anti-inflammatory response⁽⁹⁾. Recently, it has been demonstrated that caffeine exerts a beneficial effect on adipose-derived stem cells and bone marrow stromal cells by enhancing their differentiation into osteoblasts⁽¹⁰⁾. Zheng *et al.*⁽¹¹⁾ have shown that caffeine reduces the weight of intraperitoneal adipose tissues (IPAT) in mice. Yoshioka *et al.*⁽¹²⁾ have demonstrated that a high concentration of caffeine (60 mg/kg body weight) significantly increases brown adipose tissue thermogenesis and RMR and that a low concentration (40 mg/kg body weight) has no effects in mice.

These findings demonstrate that a high dose of caffeine has an anti-obesity effect and that CGA is a hypolipidaemic agent.

Abbreviations: ACO, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; ATGL, adipose TAG lipase; CAT, carnitine acyltransferase; CGA, chlorogenic acid; FAS, fatty acid synthase; IPAT, intraperitoneal adipose tissues; TC, total cholesterol.

* **Corresponding author:** Dr G. Zheng, fax +86 79183813655, email zrs150716@aliyun.com

However, studies documenting the effect of these compounds in combination on lipid metabolism are sparse. We hypothesised that a combination of caffeine and CGA might have an inhibitory effect on fat accumulation and obesity development. To test this hypothesis and identify the mechanism by which this might occur, in the present study, we investigated the effects of CGA and caffeine alone and in combination on the body weight, biochemical parameters of the liver, IPAT, and serum, and activities and mRNA and protein expression of lipid metabolism-related enzymes in mice.

Materials and methods

Ethics statement

The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Association for Laboratory Animal Science. All animal care and use protocols were approved by the Animal Care and Use Committee of Jiangxi Agricultural University. Animals were killed under anaesthesia, and every effort was made to minimise their suffering.

Animals and diets

Female ICR mice aged 4 weeks and weighing about 20 g were purchased from Animal Breeding and Research of Jiangxi Medical College (Jiangxi, China). Caffeine (>99%) was obtained from Johnson Matthey Company. CGA (>90%) was purchased from Hunan Liuyang Aite Natural Product Research and Development Company Limited.

All mice were acclimated on a standard AIN-93 G diet for 1 week. Then, forty mice were weight-matched and divided into four groups and fed diets containing no CGA or caffeine, 0.2% CGA, 0.03% caffeine, and 0.2% CGA + 0.03% caffeine. Mice were given free access to food and tap water for 24 weeks. During the feeding period, mice were weighed every week. Mice were anaesthetised and killed after the experiment, and blood was drawn from the heart and allowed to clot at room temperature. Serum was isolated by centrifugation at 825 g for 15 min at 4°C. The liver and IPAT of each mouse were harvested and weighed. Food intake was measured every day for 2 weeks. All mice were housed in an air-conditioned (temperature 24 ± 2°C and humidity 50 ± 10%) and light-controlled (12 h light–12 h dark cycle, lights on from 08.00 to 20.00 hours) animal room.

Biochemical analysis of serum and hepatic parameters

The serum concentrations of TAG, TC and glucose were determined using commercial kits (Biosino Biotechnology and Science, Inc.). The concentrations of NEFA were determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute). The serum concentration of leptin was measured using ELISA kits (R&D systems). Total lipids in the liver were extracted using the method of Folch *et al.*⁽¹³⁾. The hepatic concentrations of TAG and TC were determined using commercial kits (Biosino Biotechnology and Science,

Inc.). The concentrations of phospholipids were determined using the method of Bartlett⁽¹⁴⁾.

Measurement of the activities of hepatic lipid metabolism-related enzymes

Frozen liver samples were homogenised in buffer A (3 mM-Tris–HCl, pH 7.2, 1 mM-EDTA, 1 mM-dithiothreitol, 25 μM-ALLN (a calpain and cathepsin inhibitor, *N*-acetyl-leucyl-leucyl-norleucinal), 100 μM-leupeptin, 100 μM-AEBSF (a serine protease inhibitor, 4-(2-aminoethyl)benzenesulphonyl fluoride), 10 μM-E64 and 0.25 M-sucrose) according to the method of Moriyama *et al.*⁽¹⁵⁾. The protein concentration of the homogenate was measured and then adjusted to 10 mg/ml for carnitine acyltransferase (CAT) activity analysis. The homogenate was centrifuged at 500 g for 10 min and the supernatant was used for acyl-CoA oxidase (ACO) activity analysis. The supernatant (500 g) was further centrifuged at 9000 g for 15 min, and the resulting supernatant was used for fatty acid synthase (FAS) activity analysis. The activities of CAT, ACO and FAS were determined by the methods of Markwell *et al.*⁽¹⁶⁾, Osumi & Hashimoto⁽¹⁷⁾ and Kelley *et al.*⁽¹⁸⁾, respectively.

Measurement of the mRNA expression of lipid metabolism-related enzymes

Total RNA was isolated from frozen liver samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was obtained by reverse transcription of 1 μg of RNA using the cDNA Reverse Transcription Kit (Tanaka Biological, Inc.) according to the manufacturer's protocol. Real-time quantitative PCR was carried out using the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems) with Premix Ex Taq™ (Probe qPCR) according to the manufacturer's protocol. Briefly, PCR was carried out in a final volume of 20 μl containing 800 ng of complementary DNA, 0.4 μl of forward and reverse primers, 0.4 μl of fluorescence probe, 10 μl of Premix Ex Taq and 0.4 μl of ROX Reference Dye. PCR consisted of an initial denaturation step at 95°C for 30 s, followed by forty-five amplification cycles of 10 s at 94°C and 37 s at 60°C. The primers and probes used are given in Table 1. The results are presented as expression levels relative to those of the control after normalisation to glyceraldehyde 3-phosphate dehydrogenase using the 2^{-ΔΔC_T} method.

Measurement of the protein expression of AMP-activated protein kinase, adipose TAG lipase and fatty acid synthase in the liver

Liver tissue samples were ground in liquid N₂ and lysed in a radioimmunoprecipitation assay buffer (50 mM-Tris, pH 7.4, 150 mM-NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 2 μg/ml leupeptin) at 4°C for 1 h. Liver lysates were centrifuged at 9000 g for 15 min, and the supernatant was used for measuring the protein expression of FAS, AMP-activated protein kinase (AMPK) and adipose TAG lipase (ATGL). The protein concentration of each supernatant was determined. Equal amounts of

Table 1. Gene-specific primers and probes used in quantitative real-time PCR

Genes	Forward	Reverse	Probe
<i>ACO</i>	5'-TCACAGCAGTGGGATTCCAA-3'	5'-TCTGCAGCATCATAACA GTGTTCTC-3'	5'-TATTTACGTCACGTTTACCC GGCCTG-3'
<i>CAT</i>	5'-CTGTGGGATGGTGTATGAGCAT-3'	5'-GACATGGTCCACAAGTGCAACT-3'	5'-CAGCTGCAG AAGGGCCCCC-3'
<i>AMPK</i>	5'-TGAAGATCGGCC ACTACATCCT-3'	5'-CTTGCCACCTTCACTTTCC-3'	5'-ACACGCTTGGTGTCGGCACCTTC-3'
<i>PPARγ2</i>	5'-ATGATGGGAGAAGATAAAAT CAAGTTC-3'	5'-GGATGGCCACCTCTTTGCT-3'	5'-AACATATCACCCCCTGCAGGAGCA-3'
<i>ATGL</i>	5'-CGCGCTCTTGGCTCATG-3'	5'-CCAACCTTTGTGCCCTTAA-3'	5'-ATTCTCTTGGTGCCCATGTAGTAGCCCG-3'
<i>GADPH</i>	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-CCTGCTTACCACCTTCTTGA-3'	5'-TGCCGCTGGAGAAACCTGCC-3'

ACO, acyl-CoA oxidase; *CAT*, carnitine acyltransferase; *AMPK*, AMP-activated protein kinase; *ATGL*, adipose TAG lipase; *GADPH*, glyceraldehyde 3-phosphate dehydrogenase.

protein (30 μ g) were resolved by SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The AMPK, FAS and ATGL blots were blocked with 5% non-fat dry milk–TBST buffer (TBS containing 0.1% Tween-20) for 2 h at room temperature. The membranes were rinsed three times for 10 min each with TBST buffer and then incubated overnight at 4°C with 1:1000 dilutions of antibodies against AMPK α (no. 2793; Cell Signaling Technology, Inc.), FAS (no. 3180; Cell Signaling Technology, Inc.) and ATGL (ab85858; Abcam Limited). Equal lane loading was assessed using β -actin (Zhongshan Bio Company Limited). The blots were rinsed three times with TBST buffer for 10 min each. Washed blots were incubated with a 1:1000 dilution of a horseradish peroxidase-conjugated secondary antibody solution (ZSGB-BIO) for 2 h and washed three times with TBST buffer. The transferred proteins were visualised using an enhanced 3,3'-diaminobenzidine tetrahydrochloride kit (ZSGB-BIO).

Statistical analysis

All data are presented as means with their standard errors. Student's *t* test was used to compare the mean differences after analysis using the Data Processing System software (version 6.55, Hangzhou Reifeng Information Technology Company Limited, China; <http://www.dpsw.cn>). A *P* value <0.05 was considered significant and a *P* value <0.01 very significant.

Results

Body weight, organ weights and food intake

The effects of CGA and caffeine on the body weight, liver weight and IPAT weight of mice are summarised in Table 2. A decreasing trend was observed in the body weight of the

treatment groups. There was a significant decrease in the body weight gain of mice fed the CGA + caffeine diet ($P < 0.05$). Furthermore, IPAT weight was significantly reduced in mice fed the CGA + caffeine diet ($P < 0.05$) when compared with that in the control group. There were no significant differences in food intake between the treatment and control groups (control, CGA, caffeine and CGA + caffeine (g/d per mouse): 4.59 (SEM 0.18), 4.48 (SEM 0.32), 4.48 (SEM 0.47) and 4.62 (SEM 0.22), respectively). These results indicate that CGA + caffeine decreased the body weight and IPAT weight of mice with no effects on food intake.

Serum biochemical parameters and hepatic lipid profiles

The effects of CGA and caffeine on the serum biochemical parameters and hepatic lipid profiles of mice are summarised in Table 3. There was a significant decrease in the serum leptin concentration of mice fed the CGA + caffeine and caffeine diets ($P < 0.05$) compared with that of the control group. The serum concentrations of TC and TAG were also significantly lower in mice fed the CGA and CGA + caffeine diets than in the control group ($P < 0.05$). There was a significant decrease in the hepatic TAG concentrations of mice fed the caffeine and CGA + caffeine diets ($P < 0.05$). These results indicate that CGA + caffeine decreased serum and hepatic lipid levels.

Activities of hepatic lipid metabolism-related enzymes

The effects of CGA and caffeine on the activities of CAT, ACO and FAS in the liver of mice are shown in Fig. 1. The activity of CAT was markedly increased in mice fed the CGA + caffeine ($P < 0.01$) and caffeine ($P < 0.05$) diets than in the control

Table 2. Effects of caffeine and chlorogenic acid (CGA) on the body weight, liver weight and intraperitoneal adipose tissue (IPAT) weight of mice (Mean values with their standard errors, *n* 10)

	Control		Caffeine		CGA		CGA + caffeine	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body weight (g)	24.23	1.08	24.35	1.01	23.98	0.96	24.07	0.97
Final body weight (g)	44.67	1.38	41.75	1.46	43.24	1.42	38.36	1.17
Body weight gain (g)	20.45	1.39	17.40	1.36	19.26	1.11	14.29*	0.78
Liver (mg)	1556	81	1516	65	1518	51	1476	42
IPAT (mg)	3998	461	2875	398	4039	500	2082*	359

* Mean value was significantly different from that of the control group ($P < 0.05$).

Table 3. Effects of caffeine and chlorogenic acid (CGA) on the serum biochemical parameters and hepatic lipid profiles of mice

(Mean values with their standard errors, *n* 10)

	Control		Caffeine		CGA		CGA + caffeine	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum biochemical parameters								
TC (mmol/l)	3.20	0.18	3.28	0.27	2.78	0.16	2.61*	0.08
TAG (mmol/l)	3.84	0.44	3.46	0.67	2.41*	0.37	2.73*	0.25
GLU (mmol/l)	3.07	0.48	3.19	0.62	3.19	0.77	3.12	0.53
NEFA (μg/l)	1.50	0.29	1.57	0.27	1.39	0.21	1.48	0.31
Leptin (ng/l)	425	28	360*	20	396	29	348*	16
Hepatic lipid profiles (mg/g liver)								
TC	14.1	1.35	10.3	1.52	10.8	1.38	10.5	1.25
TAG	61.3	3.30	42.6*	3.35	49.0	5.47	37.9*	3.83
PL	15.4	0.21	15.9	0.91	14.5	0.31	14.55	0.39

TC, total cholesterol; GLU, glucose; PL, phospholipids.

* Mean value was significantly different from that of the control group ($P < 0.05$).

group. The activity of ACO was significantly increased in mice fed the caffeine and CGA + caffeine diets ($P < 0.05$), while that of FAS was significantly decreased in mice fed the CGA + caffeine diet ($P < 0.05$).

mRNA expression of lipid metabolism-related enzymes

The mRNA expression levels of lipid metabolism-related enzymes were analysed by real-time quantitative PCR, and the results are shown in Fig. 2. The mRNA expression levels of *AMPK*, *ACO*, *CAT* and *ATGL* were up-regulated by both caffeine and CGA individually and to a greater extent when used in combination. These results indicate that CGA + caffeine synergistically affected the regulation of these genes. However, the mRNA expression levels of *PPARγ2* were significantly down-regulated in mice fed the CGA and CGA + caffeine diets ($P < 0.05$), while there were no significant differences in the levels between the caffeine diet-fed and control groups.

Protein expression of AMP-activated protein kinase, adipose TAG lipase and fatty acid synthase in the liver

The protein expression levels of AMPK, ATGL and FAS were analysed by Western blotting, and the results are shown in Fig. 3. Compared with that in the control group, there was a decrease in the protein expression levels of FAS in all the treatment groups. In particular, a significant difference was observed between the CGA + caffeine diet-fed and control groups ($P < 0.05$). There was a significant increase in the protein expression levels of AMPK and ATGL in mice fed the CGA + caffeine diet ($P < 0.05$).

Discussion

In the present study, the effects of CGA + caffeine on serum and hepatic lipid levels were investigated. The results clearly showed that CGA + caffeine reduced serum and hepatic lipid levels and thus led to the suppression of body weight gain and

fat accumulation in the treated mice. Moreover, to investigate the effects of CGA + caffeine on lipid metabolism, the activities of liver metabolism-related enzymes were analysed. The results showed that CGA + caffeine increased the activities of CAT and ACO and suppressed the activity of FAS in the liver. As the liver is actively involved in β -oxidation, stimulation of lipid metabolism may contribute to the suppression of hepatic and visceral fat accumulation⁽¹⁹⁾. The activity of FAS has been reported to be positively correlated with the amount of body fat⁽²⁰⁾. The results of the present study indicate the enhancement of β -oxidation and suppression of lipogenesis to be the major reasons for the reduction of fat accumulation and body weight gain in mice.

CGA has previously been shown to significantly reduce body weight and visceral fat mass in high-fat diet-induced obese mice^(5,21). In the present study, CGA was found to have no effect on body weight or fat accumulation, which may be

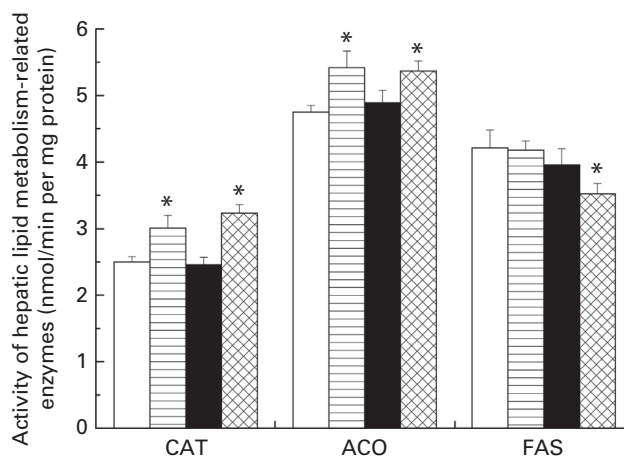


Fig. 1. Effects of chlorogenic acid (CGA) and caffeine on the activities of hepatic lipid metabolism-related enzymes. CAT, carnitine acyltransferase; ACO, acyl-CoA oxidase; FAS, fatty acid synthase. Values are means of ten mice, with their standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$). □, Control; ▤, caffeine; ■, CGA; ▨, CGA+caffeine.

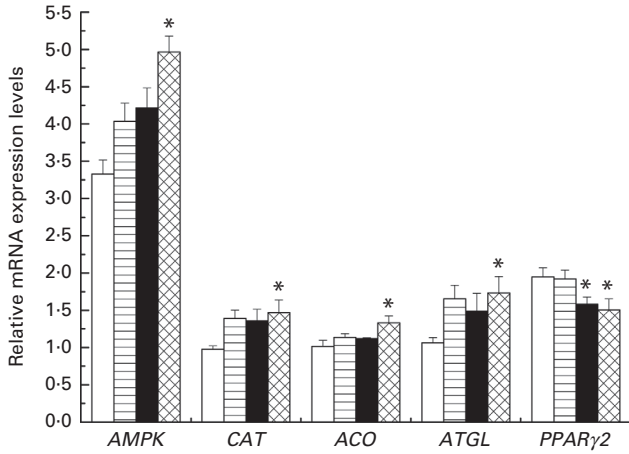


Fig. 2. Effects of chlorogenic acid (CGA) and caffeine on the hepatic mRNA expression levels of AMP-activated protein kinase (AMPK), carnitine acyl-transferase (CAT), acyl-CoA oxidase (ACO), adipose TAG lipase (ATGL) and PPAR γ 2 analysed by real-time quantitative PCR. Values are means of ten mice, with their standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$). □, Control; ▤, caffeine; ■, CGA; ▨, CGA + caffeine.

due to the normal diet rather than the high-fat diet that was used. Miura *et al.*⁽²²⁾ have reported that apple polyphenols (e.g. CGA) suppress lipid absorption and increase faecal lipid content. Caffeine has been shown to increase energy expenditure during thermogenic responses⁽²³⁾. It has also been shown that caffeine can reduce adipose tissue weight in animals^(11,12). Moreover, the intake of caffeine has been shown to elevate the serum concentrations of catecholamine in rats fed a high-fat diet⁽²⁴⁾. Tanaka *et al.*⁽²⁵⁾ postulated that caffeine enhances the degradation of fat in adipose tissues by stimulating catecholamine secretion. Some of the fatty acids that are released from adipose tissues are transported to the liver and are then oxidised. Thus, the reduced deposition of visceral fat may be related to the enhanced oxidation of fatty acids in the liver. In the present study, caffeine and CGA + caffeine were found to enhance the activity of CAT in the liver. This increased activity may be responsible for the reduction of adipose tissue weight and the suppression of body weight gain.

Leptin is produced by adipose tissues, and the serum concentrations of leptin are directly proportional to adipose tissue weight⁽²⁶⁾. Therefore, the decrease in leptin concentrations may be attributable to the reduction of IPAT weight in mice fed the CGA and caffeine diets. In mice fed the CGA + caffeine diet, leptin concentrations were significantly decreased and IPAT weight was considerably reduced, supporting the theory that the reduction of IPAT weight is related to the decrease in leptin concentrations. Neither CGA nor caffeine was found to significantly suppress body weight gain or reduce IPAT weight in the present study. However, CGA + caffeine significantly suppressed body weight gain and reduced IPAT weight. It has been shown that CGA may act synergistically with caffeine to produce anti-obesity effects.

CGA has been shown to effectively lower TC and TAG concentrations in the serum and liver⁽²¹⁾. In the present study, CGA + caffeine was found to reduce serum and hepatic TC

and TAG concentrations. The decrease in TAG concentrations in mice is thought to be induced by both the suppression of fatty acid synthesis and the acceleration of fatty acid oxidation. It has been demonstrated that caffeine elevates the activity of ACO in the liver⁽²⁴⁾. In the present study, mice were fed caffeine, CGA, and CGA + caffeine diets for 12 weeks. Both the caffeine and CGA + caffeine diets enhanced the activities of CAT and ACO in the liver, which indicates that caffeine can accelerate hepatic lipolysis by increasing the activities of ACT and ACO. In the present study, CGA + caffeine was found to inhibit the activity of FAS and down-regulate the expression of FAS protein. CGA has been shown to inhibit the activity of FAS⁽²⁷⁾, and there are a few reports that suggest that caffeine affects the activities of fatty acid synthesis enzymes. Hence, CGA in the CGA + caffeine diet may be responsible for the suppression of FAS activity in the liver.

AMPK plays an important role in the regulation of glucose and lipid metabolism^(28–30). When activated by conditions that deplete energy such as hypoxia, ischaemia and glucose deprivation, AMPK turns off ATP-consuming processes, such as fatty acid synthesis, cholesterol synthesis and gluconeogenesis, and turns on catabolic pathways that produce ATP, such as β -oxidation, glycolysis and glucose uptake⁽²⁸⁾. The activation of AMPK leads to numerous metabolic changes, which are potential targets in the treatment of metabolic

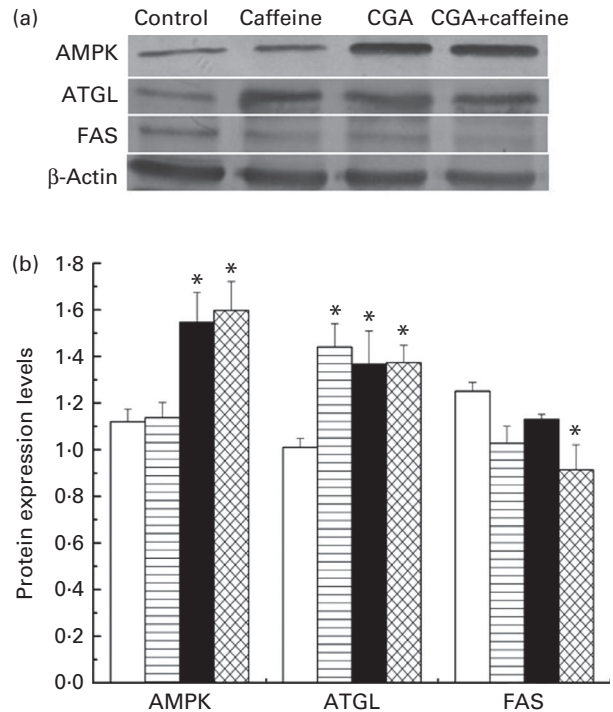


Fig. 3. Effects of chlorogenic acid (CGA) and caffeine on the hepatic protein expression levels of AMP-activated protein kinase (AMPK), adipose TAG lipase (ATGL) and fatty acid synthase (FAS). (a) Western blot analysis of AMPK, ATGL and FAS proteins isolated from the liver of mice. (b) The intensities of AMPK, ATGL and FAS protein expression levels relative to those of the control after normalisation to β -actin. Values are means of ten mice, with their standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group ($P < 0.05$). □, Control; ▤, caffeine; ■, CGA; ▨, CGA + caffeine.

disorders such as obesity, type 2 diabetes and the metabolic syndrome. In the present study, CGA + caffeine was found to significantly increase the expression of AMPK ($P < 0.05$), while caffeine or CGA alone was found to lead to upward trends, but these were unremarkable. Interestingly, the mRNA expression levels of *AMPK*, *ACO* and *CAT* were significantly up-regulated by CGA + caffeine, which indicates that the increased expression of *AMPK* might have promoted the expression of *ACO* and *CAT*, which are the main contributors of β -oxidation. To confirm these findings, the expression of AMPK was measured and CGA + caffeine was found to significantly increase the protein expression levels of AMPK. ATGL is highly expressed in white adipose tissue and is the predominant TAG hydrolase in mammals, and although its role in the liver is largely unknown, it does appear to be a major hepatic TAG lipase⁽³¹⁾. Together with hormone-sensitive lipase, it accounts for more than 90% of TAG hydrolase activity in the white adipose tissue of mice⁽³²⁾. The activity of TAG hydrolase in the white adipose tissue of ATGL^{-/-} mice is 80% lower than that in the tissue of wild-type mice, suggesting that ATGL is the rate-limiting enzyme in TAG hydrolysis in white adipose tissue, whereas hormone-sensitive lipase functions primarily as a diglyceride hydrolase⁽³³⁾. In the present study, the expression of *ATGL* mRNA was increased by both caffeine and CGA, but CGA + caffeine yielded more dramatic results. Western blotting showed that caffeine, CGA and CGA + caffeine up-regulated the protein expression levels of ATGL considerably, which indicates that CGA + caffeine may contribute to lipolysis. Further investigation is required to determine whether the increased AMPK levels promoted the expression of ATGL. AMPK is activated by two distinct pathways, an AMP-dependent pathway mediated by liver kinase B1 and a Ca^{2+} -dependent pathway mediated by $\text{Ca}(2+)/\text{CaM}$ -dependent protein kinase kinase- β ⁽²⁹⁾. The activation of AMPK depends on the phosphorylation of Thr172 on its α -subunit, which leads to reduced energy storage and increased energy production to re-establish normal cellular energy balance⁽³⁰⁾. Binding of AMP to the γ -subunit leads to the allosteric activation of AMPK, as well as to the protection of Thr172 from dephosphorylation, thereby keeping the enzyme activated. Further studies on the relationship between phosphorylated AMPK and *ACO*, *CAT* and *ATGL* are required to fully evaluate this mechanism.

PPAR γ is a nuclear receptor with many diverse functions, including the regulation of genes associated with proliferation and differentiation, in a variety of cell types. Its most remarkable function is the regulation of adipose tissue development, which involves coordination of the expression of thousands of genes responsible for the establishment of the mature adipocyte phenotype⁽³⁴⁾. Many investigations have demonstrated that PPAR γ 2 is a potential physiological sensor of lipid levels, linking fatty acids and other lipid-related molecules to glucose and lipid homeostasis. PPAR γ 2 regulates the expression of adipogenic genes, is expressed selectively in adipose tissues, and promotes the differentiation and proliferation of adipocytes, leading to an increase in adipose tissue mass^(35,36). In the present study, the mRNA expression levels of *PPAR* γ 2 were significantly down-regulated in the liver of

mice fed the CGA and CGA + caffeine diets, while no remarkable regulating effects were observed in mice fed the caffeine diet, indicating that caffeine enhanced a CGA-mediated decrease in *PPAR* γ 2 mRNA expression levels and then contributed to lipolysis. Similar to PPAR γ , CCAAT enhancer-binding protein- α is also known to be a key transcription factor for gene activation and differentiation. Both proteins are important for lipogenesis at the molecular level, because they cross-regulate each other's expression as well as govern the expression of the entire adipogenic programme, which includes the activation of additional transcription factors⁽³⁷⁾.

It might be interesting to equate the doses of caffeine and CGA used in the present study with those found in a typical cup of coffee. This is not entirely straightforward as these values vary dramatically depending on the type of coffee and the preparation method⁽³⁸⁾. Typical values in green coffee bean are of the order of 8% for CGA and 1.2% for caffeine, so the doses used in the present study are not too far removed from the daily consumption levels. The dose of caffeine used in the present study approximately equals that present in two cups of coffee.

In summary, the results of the present study demonstrate that CGA + caffeine affects the activities of lipid metabolism-related enzymes through the regulation of their mRNA and protein expression levels. The enhancement of fatty acid oxidation and suppression of FAS activity were found to decrease serum and hepatic lipid levels and then suppress fat accumulation and body weight gain. The up-regulated *AMPK* mRNA and protein expression may promote lipolysis. The results also indicate that it might be possible to prevent obesity by continuous and long-term administration of CGA and caffeine together. In light of such beneficial effects, the coffee bean has potential as a functional food that can prevent the onset of lifestyle-related diseases.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (no. 31160320) and sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholar, State Education Ministry (2009). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors' contributions are as follows: G. Z. conceived and designed the experiments; Y. Q. and D. L. carried out the experiments; G. Z., Q.-F. Z. and D. L. analysed the data, G. Z. and Y. Q. wrote the article.

None of the authors has any conflicts of interest to declare.

References

- Zhang A, Sun H & Wang X (2012) Power of metabolomics in biomarker discovery and mining mechanisms of obesity. *Obes Rev* **14**, 344–349.
- Monteiro M, Farah A, Perrone D, *et al.* (2007) Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. *J Nutr* **137**, 2196–2201.
- Narita Y & Inoue K (2013) Degradation kinetics of chlorogenic acid at various pHs and effect of ascorbic acid and epigallocatechin gallate on its stability under alkaline conditions. *J Agric Food Chem* **61**, 966–972.

4. Ong KW, Hsu A & Tan BKH (2012) Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. *PLOS ONE* **7**, e32718.
5. Cho AS, Jeon SM, Kim MJ, *et al.* (2010) Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food Chem Toxicol* **48**, 937–943.
6. Hirata T, Kobayashi T, Wada A, *et al.* (2011) Anti-obesity compounds in green leaves of *Eucommia ulmoides*. *Bioorg Med Chem Lett* **21**, 1786–1791.
7. Hsu CL, Hung SL & Yen GC (2006) Inhibitory effect of phenolic acids on the proliferation of 3T3-L1 preadipocytes in relation to their antioxidant activity. *J Agric Food Chem* **54**, 4191–4197.
8. Greenberg JA, Boozer CN & Geliebter A (2006) Coffee, diabetes, and weight control. *Am J Clin Nutr* **84**, 682–693.
9. Tauler P, Martinez S & Moreno C (2013) Effects of caffeine on the inflammatory response induced by a 15-km run competition. *Med Sci Sport Exerc* **45**, 1269–1276.
10. Su SJ, Chang KL, Su SH, *et al.* (2013) Caffeine regulates osteogenic differentiation and mineralization of primary adipose-derived stem cells and a bone marrow stromal cell line. *Int J Food Sci Nutr* **64**, 429–436.
11. Zheng G, Sayama K, Okubo T, *et al.* (2004) Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. *In vivo* **18**, 55–62.
12. Yoshioka K, Yoshida T, Kamanaru K, *et al.* (1990) Caffeine activates brown adipose tissue thermogenesis and metabolic rate in mice. *J Nutr Sci Vitaminol* **36**, 173–178.
13. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 479–509.
14. Bartlett GR (1959) Colorimetric assay methods for free and phosphorylated glyceric acids. *J Biol Chem* **234**, 469–471.
15. Moriyama T, Kishimoto K, Nagai K, *et al.* (2004) Soybean beta-conglycinin diet suppresses serum triglyceride levels in normal and genetically obese mice by induction of beta-oxidation, down regulation of fatty acid synthase, and inhibition of triglyceride absorption. *Biosci Biotechnol Biochem* **68**, 353–359.
16. Markwell MA, McGroarty EJ, Bieber LL, *et al.* (1973) The subcellular distribution of carnitine acyltransferases in mammalian liver and kidney. *J Boil Chem* **248**, 3426–3432.
17. Osumi T & Hashimoto T (1978) Acyl-CoA oxidase of rat liver: a new enzyme for fatty acid oxidation. *Biochem Biophys Res Commun* **83**, 479–485.
18. Kelley DS, Nelson GJ & Hunt JE (1986) Effect of prior nutritional status on the activity of lipogenic enzymes in primary monolayer cultures of rat hepatocytes. *Biochem J* **235**, 87–90.
19. Murase T, Nagasawa A, Suzuki J, *et al.* (2002) Beneficial effect of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int J Obes* **26**, 1459–1464.
20. Li M, Shi Y & Tian W (1999) Factor influencing the levels of fatty acid synthase complex activity in fowl. *Biochem Mol Biol Int* **47**, 63–69.
21. Peng CH, Liu LK, Chuang CM, *et al.* (2011) Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *J Agric Food Chem* **59**, 2663–2671.
22. Miura D, Miura Y & Yagasaki K (2007) Effect of apple polyphenol extract on hepatoma proliferation and invasion in culture and on tumor growth, metastasis, and abnormal lipoprotein profiles in hepatoma-bearing rats. *Biosci Biotechnol Biochem* **71**, 2743–2750.
23. Riedel A, Pignitter M, Hochkogler CM, *et al.* (2012) Caffeine dose-dependently induces thermogenesis but restores ATP in HepG2 cells in culture. *Food Funct* **3**, 955–964.
24. Kobayashi-Hattori K, Mogi A, Matsumoto Y, *et al.* (2005) Effect of caffeine on the body fat and lipid metabolism of rats fed on a high-fat diet. *Biosci Biotechnol Biochem* **69**, 2219–2223.
25. Tanaka K, Tamaru S, Nishizono S, *et al.* (2010) Hypotriacylglycerolemic and antiobesity properties of a new fermented tea product obtained by tea-rolling processing of third-crop green tea (*Camellia sinensis*) leaves and loquat (*Eriobotrya japonica*) leaves. *Biosci Biotechnol Biochem* **74**, 1606–1612.
26. Janeckova R (2001) The role of leptin in human physiology and pathophysiology. *Physiol Res* **50**, 443–459.
27. Li BH, Ma XF, Wu XD, *et al.* (2006) Inhibitory activity of chlorogenic acid on enzymes involved in the fatty acid synthesis in animals and bacteria. *IUBMB Life* **58**, 39–46.
28. Lee CE, Hur HJ, Hwang JT, *et al.* (2012) Long-term consumption of Platycodi radix ameliorates obesity and insulin resistance via the activation of AMPK pathways. *Evid Based Complement Alternat Med* **2012**, 759143.
29. Sanders MJ, Grondin PO, Hegarty BD, *et al.* (2007) Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem J* **403**, 139–148.
30. Zhang BB, Zhou G & Li C (2009) AMPK: an emerging drug target for diabetes and the metabolic syndrome. *Cell Metab* **9**, 407–416.
31. Ong KT, Mashek MT, Bu SY, *et al.* (2011) Adipose triglyceride lipase is a major hepatic lipase that regulates triacylglycerol turnover and fatty acid signaling and partitioning. *Hepatology* **53**, 116–126.
32. Lord CC & Brown JM (2012) Distinct roles for alpha-beta hydrolase domain 5 (ABHD5/CGI-58) and adipose triglyceride lipase (ATGL/PNPLA2) in lipid metabolism and signaling. *Adipocyte* **1**, 123–131.
33. Haemmerle G, Lass A, Zimmermann R, *et al.* (2006) Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* **312**, 734–737.
34. Farmer SR (2005) Regulation of PPAR γ activity during adipogenesis. *Int J Obes* **29**, S13–S16.
35. Otto TC, Lane MD & Cox MM (2005) Adipose development: from stem cell to adipocyte. *Crit Rev Biochem Mol Biol* **40**, 229–242.
36. Gustafson B & Smith U (2006) Cytokines promote Wnt signaling and inflammation and impair the normal differentiation and lipid accumulation in 3T3-L1 preadipocytes. *J Biol Chem* **281**, 9507–9516.
37. Wu Z, Rosen ED, Brun R, *et al.* (1999) Cross-regulation of C/EBP α and PPAR γ controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell* **3**, 151–158.
38. Niseteo T, Komes D, Belščak-Cvitanović A, *et al.* (2012) Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. *Food Chem* **134**, 1870–1877.