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Supplementation of a High-Fat Diet with Chlorogenic Acid Is Associated with Insulin Resistance and Hepatic Lipid Accumulation in Mice

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ABSTRACT: The increasing prevalence of the metabolic syndrome requires a greater need for therapeutic and prevention strategies. Higher coffee consumption is consistently associated with a lower risk of type 2 diabetes in population studies. Dietary polyphenols have been linked to benefits on several features of the metabolic syndrome. Chlorogenic acid (CGA), a major component of coffee, is one of the most consumed polyphenols in the diet. In our study, we conducted a controlled dietary intervention over 12 weeks in male mice. There were three dietary groups: (i) normal diet, (ii) high-fat diet, and (iii) high-fat diet + CGA. We assessed the effect of CGA at a physiologically obtainable dose (1 g/kg of diet) on high-fat-diet-induced obesity, glucose intolerance, insulin resistance, and also fatty acid oxidation and insulin signaling in C57BL/6 male mice. Supplementation of CGA in the high-fat diet did not reduce body weight compared to mice fed the high-fat diet alone ($p = 0.32$). CGA resulted in increased insulin resistance compared to mice fed a high-fat diet only ($p < 0.05$). CGA resulted in decreased phosphorylation of AMP-activated protein kinase (AMPK) ($p < 0.001$) and acetyl carboxylase β (ACCβ), a downstream target of AMPK ($p < 0.05$), in liver. The liver of mice fed a high-fat diet supplemented with CGA had a higher lipid content ($p < 0.05$) and more steatosis relative to mice fed a high-fat diet only, indicating impaired fatty acid oxidation. This study suggests that CGA supplementation in a high-fat diet does not protect against features of the metabolic syndrome in diet-induced obese mice.

KEYWORDS: *Chlorogenic acid, glucose tolerance, insulin sensitivity, fatty acid oxidation, mouse, metabolic syndrome*

INTRODUCTION

The metabolic syndrome consists of a group of metabolic abnormalities, such as abdominal obesity, dyslipidemia, hyperglycemia, and hypertension.[1] The condition is associated with the increased risk of developing cardiovascular disease and type 2 diabetes.[1,2] Emergence of metabolic syndrome incidence has resulted in an increased need for therapeutic and prevention strategies. Lifestyle changes[3] and potential treatment that targets specific molecules for regulation of metabolic pathways[4] are recommended approaches toward managing metabolic syndrome.

Epidemiological studies have shown inverse associations between coffee consumption and risk of developing chronic diseases, such as type 2 diabetes mellitus[5] and cancer.[6] These potential benefits are also indicated in individuals consuming decaffeinated coffee.[5,7] This suggests that compounds in coffee other than caffeine may be responsible for the health benefits.

Numerous studies suggest health benefits of polyphenols. They include reducing blood pressure,[8] improvement of endothelial function and nitric oxide status,[9] and augmentation of insulin sensitivity.[10] Chlorogenic acid (CGA), is an ester of caffeic acid and quinic acid.[11] This compound is rich in coffee, as well as other sources, such as in fruits like plums,[12] apples, and berries.[13] Coffee consumers can obtain up to 1 g of CGA from daily consumption.[11] In a coffee-drinking population, CGA is likely to be one of the major polyphenols in the diet.[14] Coffee polyphenols have been shown to have various health-protective effects, such as suppressing body fat accumulation,[15] reducing liver damage,[16] and inhibiting hyperglycaemia, hyperinsulinemia, and hyperlipidemia.[17] However, these studies either looked at coffee polyphenols, which includes a combination of many phenolic acids, including CGA and ferulic acid,[15] or coffee extract, which may have been a combined effect from the various compounds found in coffee. A recent study that looked at specific coffee polyphenols has reported that caffeic acid stimulates AMP-activated protein kinase (AMPK) activity in rat skeletal muscle and enhances insulin-independent glucose transport, while none of the effects were seen with CGA treatment.[18] However, effects of pure

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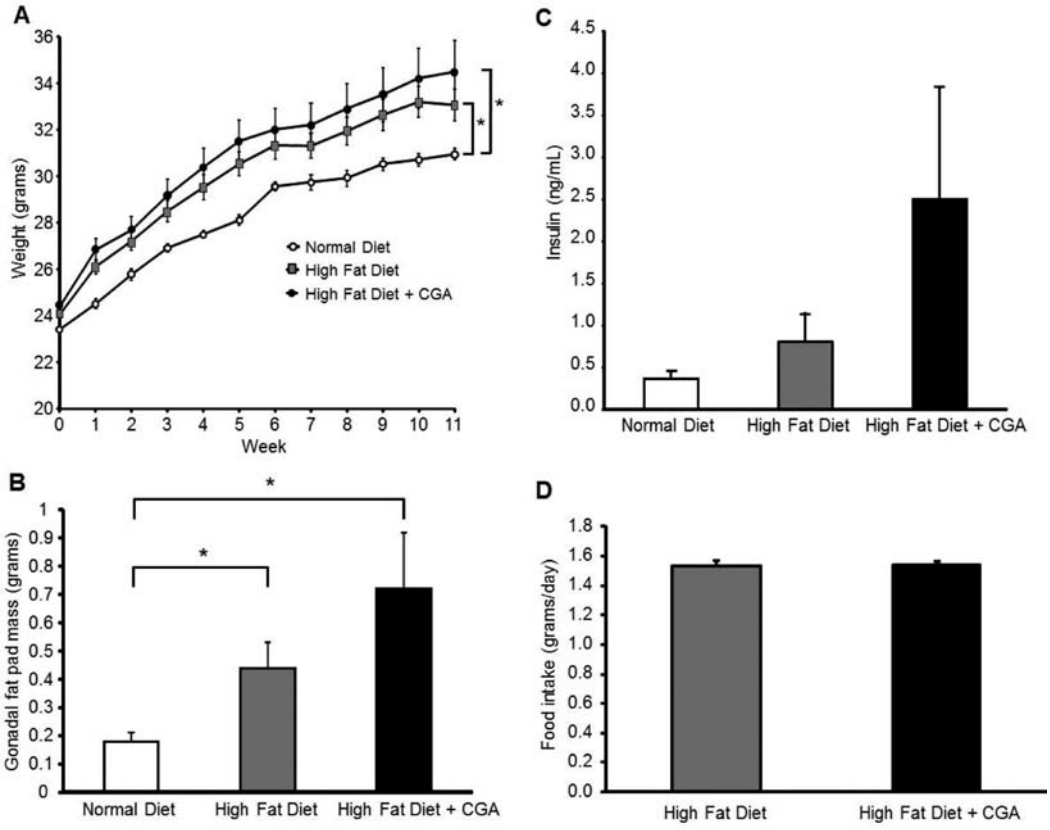

Figure 1. High-fat-diet-induced weight gain with CGA supplementation. (A) Weight gain in mice fed a normal diet (white circle), high-fat diet (gray square), and high-fat diet + CGA (black circle), (B) gonadal fat pad weights, (C) fasting insulin measurements at week 12, and (D) food intake. Data are expressed as the mean ± SEM; n = 10 mice per group for A and D and n = 5 for B and C. (*) $p < 0.05$.

CGA, which is the most abundant form of phenolic acid in coffee, on risks of metabolic disorders remain to be fully elucidated.

The aim of the present study was to test the effect of CGA on high-fat-diet-induced obesity, glucose intolerance, and insulin resistance in mice. We also assessed the effects of CGA on fatty acid oxidation and insulin signaling.

MATERIALS AND METHODS

Experimental Animal and Diets. Male C57BL/6 mice were obtained from the Animal Resources Centre, Murdoch, Western Australia, Australia, and were maintained on a normal diet (14.3 MJ/kg, 76% of energy from carbohydrate, 5% from fat, and 19% from protein; Specialty Feeds, Glen Forrest, Western Australia, Australia) until 7 weeks of age. The mice were allowed to acclimatize to conditions in the animal holding room, and they were maintained on a 12 h light/dark cycle. Mice were then switched to 3 groups of different diets (n = 10 in each group, 5 mice/cage), which are a normal diet, a high-fat diet (19 MJ/kg, 36% of energy from carbohydrate, 43% of energy from fat, and 21% from protein; Specialty Feeds), and a high-fat diet that contained 1 g of CGA/kg of diet (Atlantic SciTech Group, Linden, NJ) for a further 12 weeks. Water and feed were available to the animals *ad libitum*. All surgery was performed under methoxyflurane anesthesia, and all efforts were made to minimize suffering.

Ethics Statement. All animal experiments were approved by the Royal Perth Hospital Animal Ethics Committee (approval number R510/11-12).

Body Weight, Adiposity, and Basal Insulin Measurement. Body weights of mice were measured weekly throughout the study. Gonadal fat pad mass was also measured at the time of sacrifice. Mice were fasted for 5 h prior to sacrifice. Fasting blood samples were collected via cardiac puncture, and serum was obtained. From this sample, insulin was measured using standard enzyme-linked immunosorbent assay (ELISA) kits as previously described.[19]

Metabolic Assays. Glucose tolerance tests (GTTs) were performed at weeks 5 and 10 of the experiment, while insulin tolerance tests (ITTs) were performed at weeks 6 and 11 of the experiment. Food was withdrawn from mice 5 h prior to testing. Blood samples were obtained by tail bleeding, and glucose levels were measured using a glucometer (AccuCheck II; Roche, Castle Hill, New South Wales, Australia) immediately before and at 15, 30, 45, 60, 90, and 120 min after an intraperitoneal injection of glucose (1 g/kg of body mass) or insulin (0.5 unit/kg of body mass).

Food Intake. Food was weighed manually for mice fed either a high-fat diet only or a high-fat diet with CGA supplementation for 11 weeks. The grams of diet consumed per mouse were then determined per day.

Acute Insulin Signaling Experiment. For insulin signaling experiments, mice were anaesthetized with methoxyflurane before an intraperitoneal injection with insulin (2 units/kg) or saline. Subsequently, liver, adipose tissue, and skeletal muscle samples were obtained within 5 min of injection and snap-frozen in liquid nitrogen, before being analyzed for protein concentration.

Liver and Adipose Tissue Histology. At the end of the experiment, white adipose tissue and liver were dissected and fixed in 4% paraformaldehyde (w/w) 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, before being mounted using DePeX (Sigma-Aldrich, St. Louis, MO). Sections were then visualized and photographed using the Olympus 1X71 microscope (Olympus, Mt. Waverly, Victoria, Australia).

Hepatic Lipid Analysis. Total lipid from the liver was extracted with a chloroform–methanol mixture (2:1) according to the method by Folch et al.[20] Empty glass tubes were weighed prior to the

extraction process. After completing the extraction process, extracts were dried under vacuum using a sample concentrator (miVac, Ipswich, U.K.) and then the tubes were reweighed to determine the weight of lipid extracted from the liver samples.

Western Blot Analysis of Proteins Associated with Fatty Acid Oxidation and Insulin Signaling. Liver, white adipose tissue, and skeletal muscle (quadricep) were dissected from mice on the final day of the experiment and were snap-frozen in liquid nitrogen. Tissue samples were lysed by homogenization in cytostolic extraction buffer containing protease and phosphatase inhibitors. The lysates were then centrifuged at 15493*g* for 10 min at 4 °C; supernatants were collected; and the protein concentration was determined using BCA Protein Assay Reagent (bicinchoninic acid; Thermo Scientific, Hanover Park, IL). Samples were then separated with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and western blotting was carried out to detect total AKT, phosphorylated AKT (Ser^{473}), phosphorylated acetyl carboxylase β (ACCβ Ser^{79}), and phosphorylated AMP-activated protein kinase (AMPK Thr^{172}) using primary antibodies from Cell Signaling Technology (Danvers, MA).[21,22] The β-actin antibody was obtained from AbCam. Horseradish-peroxidase-conjugated secondary antibodies were used, and protein bands were detected by chemi-illuminescence using the Multi Image II (Alpha Innotech).

Statistical Analyses. Statistical analyses were performed using IBM SPSS Statistics 19 (IBM Corporation, Armonk, NY) and SAS 9.3 (SAS Institute, Inc., Cary, NC). Results are expressed as the mean ± standard error of the mean (SEM). Mean values were compared for differences by analysis of variance with Tukey's adjustment for multiple comparisons (three-group analyses) or Student's *t* test for unpaired samples (two-group analyses). $p < 0.05$ was considered to be statistically significant. For data with repeated measures over time (weight, GTT, and ITT), random-effect linear models were fitted in SAS using the PROC MIXED command to observed data for each variable (weight and glucose). The mouse number was included as a random factor in all models. The models also contained fixed effects for treatment group (diet) and time as a categorical variable.

RESULTS

Effect of CGA Supplementation on High-Fat-Diet-Induced Obesity and Insulin Levels in C57BL/6 Mice. The high-fat diet and high-fat diet + CGA groups both displayed a higher rate and absolute body weight increase compared to the normal diet group (Figure 1A). The rate and absolute body weight increase did not differ between the two high-fat diet groups. Mice fed the high-fat diet or high-fat diet + CGA also displayed increased gonadal fat pad mass (Figure 1B) compared to the mice fed the normal diet. Differences in gonadal fat pad mass between the two high-fat diet groups were not significant ($p = 0.23$). Both body weight and gonadal fat pad measurements confirmed that the high-fat diet induced obesity in C57BL/6 mice.

We measured fasting insulin levels in basal serum of mice using ELISA. A trend for hyperinsulinemia was evident in the high-fat diet + CGA fed mice (Figure 1C) compared to high-fat diet fed mice and normal diet fed mice. All subsequent analysis presented in this paper will now compare high-fat diet fed mice to the high-fat diet fed mice supplemented with CGA.

We also measured the estimate of food intake in both high-fat diet fed mice and high-fat diet + CGA fed mice (Figure 1D). Food intake was similar between the two groups of mice.

CGA Supplementation Promoted Glucose Intolerance and Insulin Resistance. We performed intraperitoneal GTT (at weeks 5 and 10) and ITT (at weeks 6 and 11) after commencement of feeding (panels A and B of Figure 2 and panels A and B of Figure 3). A tendency for glucose intolerance was observed with CGA supplementation compared to the high-fat diet alone, particularly at week 10 (Figure 2B). Interestingly, we demonstrated that the CGA supplementation group was markedly more insulin-resistant compared to the high-fat diet alone mice ($p < 0.05$), and this increased with the duration of the diet (panels A and B of Figure 3). We conducted systematic analysis of insulin stimulation in a number of metabolic tissues to assess in which compartment insulin resistance was prevailing with CGA supplementation.

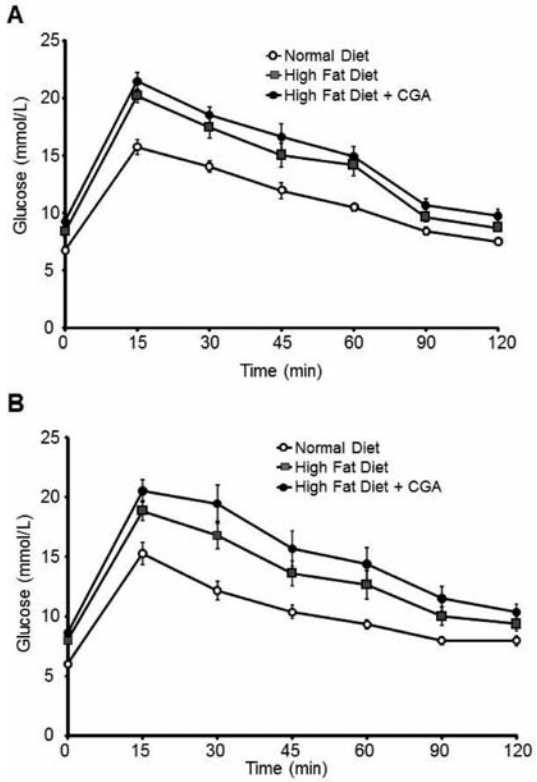

Figure 2. CGA supplementation induced a tendency for glucose intolerance in mice. GTT at (A) week 5 and (B) week 10 in normal diet (white circle), high-fat diet (gray square), and high-fat diet + CGA (black circle) fed mice. Data are expressed as the mean ± SEM; n = 9−10 mice per group.

CGA Resulted in Mild Insulin Resistance in White Adipose Tissue. Western blot analysis of insulin signaling in white adipose tissue confirmed reduced phosphorylation of Akt (Ser^{473}) with CGA supplementation (Figure 4A). High-fat diet fed mice showed a 10.8-fold increase in insulin-induced AKT phosphorylation compared to the CGA supplemented group, which had a 6.9-fold increase in insulin-induced AKT phosphorylation. However, the difference was not significant.

When assessing insulin stimulation in liver, we did not observe an effect as a result of CGA supplementation. This suggests that CGA-induced insulin resistance is not occurring in the liver (Figure 4B). Both groups of mice showed a 1.8-fold increase in insulin-induced AKT phosphorylation. We also assessed insulin-induced AKT phosphorylation in skeletal muscle (quadricep) (Figure 4C). From this analysis, we found no effect of CGA supplementation on insulin signaling in the skeletal muscle (quadricep), where the high-fat diet showed a 4.4-fold increase in insulin-induced AKT phosphorylation, while the CGA supplemented group displayed a 5.5-fold increase in insulin-induced AKT phosphorylation. This suggests that CGA-induced insulin resistance did not occur in the quadricep.

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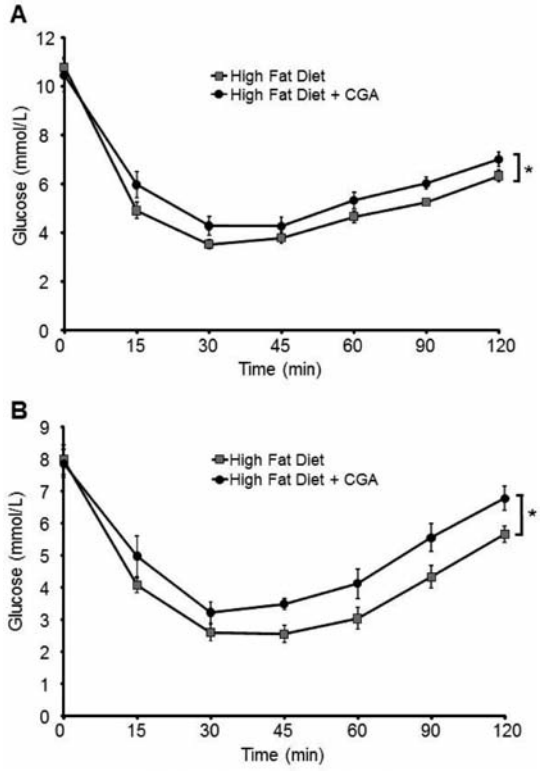

Figure 3. CGA supplementation induced insulin resistance in mice. ITT at (A) week 6 and (B) week 11 in high-fat diet (gray square) and high-fat diet + CGA (black circle) fed mice. Data are expressed as the mean ± SEM; n = 9−10 per group. (∗) $p < 0.05$.

CGA Supplementation Reduced Fatty Acid Oxidation Signaling in Liver and White Adipose Tissue. We examined the phosphorylation of AMPK and ACCβ as markers of fatty acid oxidation in a number of metabolic tissues. Analysis of liver highlighted that phosphorylation of AMPK and its downstream target ACCβ was decreased in liver with CGA supplementation (Figure 5). This result suggests that decreased fatty acid oxidation prevailed in the mice after CGA supplementation. We examined liver histology using hematoxylin and eosin staining. Interestingly, we observed markedly more steatosis in the liver of high-fat fed mice supplemented with CGA (Figure 6B) compared to high-fat fed only mice (Figure 6A). Moreover, the hepatic lipid content was higher in mice supplemented with CGA compared to mice fed a high-fat diet alone (Figure 6C). This is consistent with the reduced phosphorylation of AMPK and ACCβ seen in the same group of mice, which suggests an impaired fatty acid oxidation pathway.

Fatty acid oxidation signaling was also assessed in white adipose tissue. In this tissue, CGA supplementation resulted in a tendency for decreased phosphorylation of ACCβ, the downstream target of AMPK, which suggests decreased fatty acid oxidation in white adipose tissue of mice fed a high-fat diet supplemented with CGA (Figure 7). Finally, assessment of white adipose tissue by hematoxylin and eosin staining revealed adipocyte hypertrophy in mice fed a high-fat diet supplemented with CGA compared to mice fed a high-fat diet alone (Figure 8).

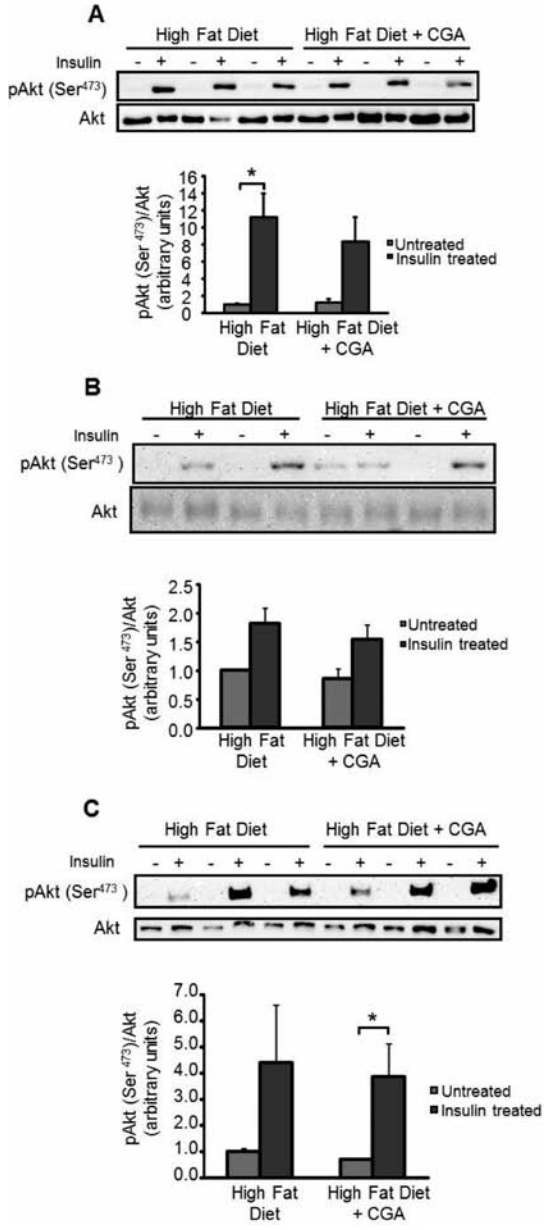

Figure 4. Effect of CGA supplementation on insulin sensitivity in white adipose tissue, liver, and skeletal muscle. Representative immunoblots of total and phosphorylated (Ser^{473}) Akt and quantification of insulin-stimulated phosphorylation of Akt (Ser^{473}) in (A) white adipose tissue, (B) liver, and (C) skeletal muscle. Data are expressed as the mean ± SEM; n = 4 mice per group. (∗) $p < 0.05$.

DISCUSSION

We sought to assess the effect of CGA supplementation on diet-induced obesity, glucose intolerance, insulin resistance, and signaling pathways associated with glucose homeostasis and fatty acid oxidation in mice. Our results do not support the hypothesis that CGA can prevent development of features of the metabolic syndrome.

We demonstrated that 12 weeks of high-fat feeding induced an increase in body weight and hyperinsulinemia in C57BL/6J mice. The same observation has previously been seen by our group[23] and others.[24,25] Interestingly, a high-fat diet supplemented with CGA increased body weight gain compared to mice fed a normal diet. Mice fed a high-fat diet only and a high-

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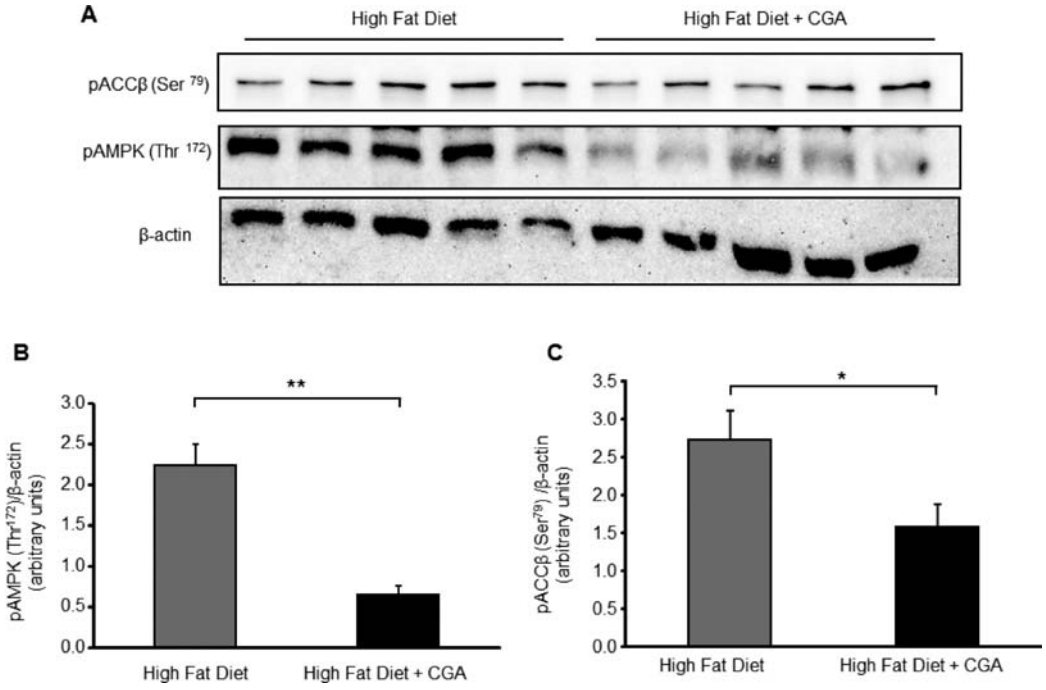

Figure 5. CGA supplementation decreases AMPK signaling in the liver. (A) Representative immunoblots and quantification of phosphorylation of (B) AMPK (Thr^{172}) and (C) ACCβ (Ser^{79}) in liver. Data are expressed as the mean ± SEM; n = 5 mice per group. (*) $p < 0.05$ and (**) $p < 0.001$.

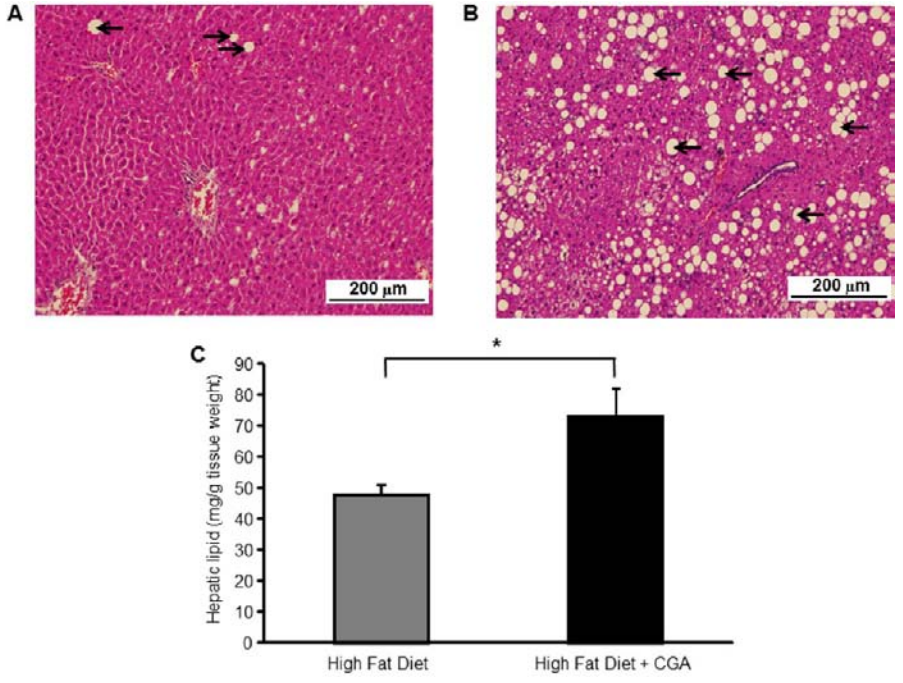

Figure 6. Effect of CGA supplementation on high-fat-diet-induced steatosis and lipid content in liver. Representative hematoxylin and eosin staining of liver of mice fed a (A) high-fat diet and (B) high-fat diet + CGA and (C) lipid quantitation. Arrows indicate steatosis. Data are expressed as the mean ± SEM; n = 10 per group. (*) $p < 0.05$.

fat diet with CGA supplementation showed similar body weights. Despite the similar body weight, fasting insulin was further elevated in mice fed a high-fat diet supplemented with CGA compared to mice fed a high-fat diet alone. Gonadal fat pad mass was also increased 2.5-fold in mice fed a high-fat diet compared to those fed a normal diet and was 4-fold higher in mice fed a high-fat diet supplemented with CGA when compared to those fed a normal diet. These results are not consistent with the study by Cho et al.,[26] who observed that CGA and its metabolite, caffeic acid, reduced weight gain and visceral fat mass in high-fat-diet-induced obese imprinting control region (ICR) mice. A recent study by Murase et al.[17] observed that coffee polyphenols suppressed body fat accumulation in high-fat-diet-induced obese mice.[17] It should be noted that our study used a higher dose of CGA (1 g/kg of diet) compared to the study by Cho et al.[26] (0.2 g/kg of diet).

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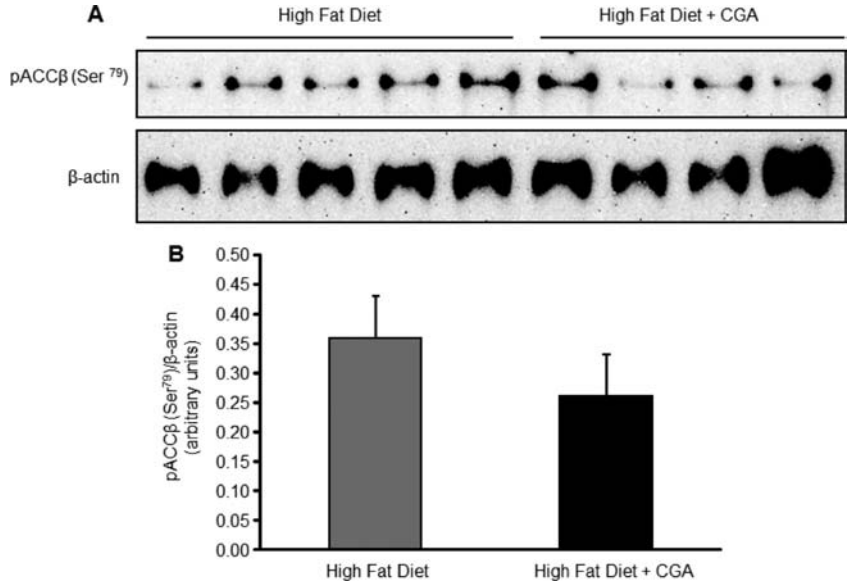

Figure 7. CGA supplementation reduces phosphorylation of ACCβ in white adipose tissue. (A) Representative immunoblots and (B) quantification of phosphorylation of ACCβ (Ser^{79}) in white adipose tissue. Data are expressed as the mean ± SEM; n = 4−5 mice per group.

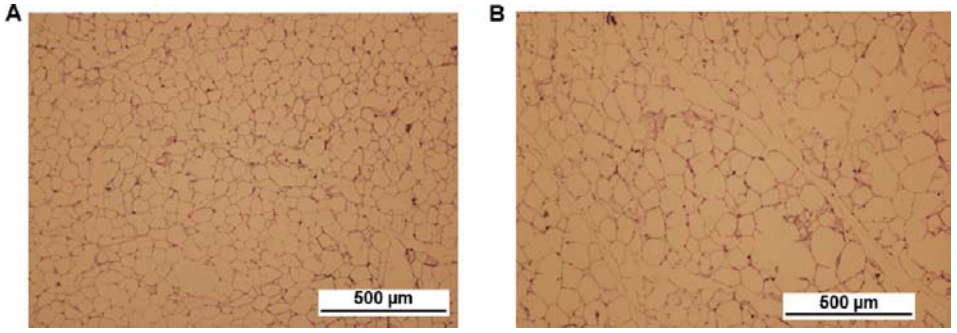

Figure 8. CGA supplementation promotes adipocyte hypertrophy. Representative hematoxylin and eosin staining of white adipose tissue of mice fed a (A) high-fat diet and (B) high-fat diet + CGA.

Cho et al.[26] employed a different strain of mice in their study. These differences could explain some of the variation in results compared to our study.

We also measured food intake in the mice fed either a high-fat diet only or a high-fat diet with CGA supplementation. Our results showed that there was no difference in food intake between the two groups (Figure 1D). Unfortunately, we did not collect feces in our current studies. Future studies should collect feces from mice fed either a high-fat diet only or a high-fat diet with CGA supplementation to compare the microbiota between the two groups of mice. These data may support a role for bacterial pathogens in the phenotypical differences observed between mice fed either a high-fat diet only or a high-fat diet with CGA supplementation. These future studies are vital because the CGA in the diet will be degraded by the action of the local microbiota to give rise to small bioactive phenolic acid and aromatic catabolites in the circulation.[27] Colonic metabolites of CGAs include *m*-coumaric acid, dihydroferulic acid, dihydrocaffeic acid, and feruloylglycine. These metabolites may be responsible for the observed phenotype, or they may work in concert.

Our study is not the first to demonstrate that higher doses of a particular compound or chemical may promote detrimental effects on health. A long-term French study demonstrated that moderate wine drinkers (<60 g of alcohol/day) had lower risks of deaths from all causes at all levels of systolic blood pressure. In this same study, there was no significant reduction in all-cause mortality in heavier drinkers.[28]

When determining the dose of a compound to administer to mice, it is vital to ensure that it will ultimately equate to physiological concentrations in humans. It has been highlighted that it would be naive to just use body weight to extrapolate safe doses from animals to humans. It has been suggested that using body surface area would allow for better estimates because the allometric scaling factor at least approximates that of clearance.[29] On the basis of body surface area, the dose in humans should be 405 greater than the dose in mice. Hence, in our current study, a single mouse would consume approximately 3 mg of CGA/day. On the basis of body surface area, this equates to 1215 mg of CGA in humans/day. This is a physiological dose in humans because a single cup of coffee contains approximately 250 mg of CGA (1215 mg of CGA = 5 cups of coffee).

To our knowledge, this is the first study of the effect of CGA on glucose tolerance and insulin sensitivity in high-fat-diet-induced obese mice. We did not observe any improvement in glucose tolerance in mice fed a high-fat diet supplemented with CGA. This is not in accordance with the study by Bassoli et al.,[30] who observed a reduced plasma glucose peak in the oral GTT following an acute oral administration of CGA at 3.5 mg/

kg of body weight in rats. Ong et al.[31] also found a reduction of the blood glucose level after intraperitoneal administration of 250 mg/kg of CGA in *db/db* mice. The different methods of CGA administration and dose used in the experiment may explain some of the variations in results compared to our study. We believe that performing the GTT and ITT on mice fed a high-fat diet supplemented with CGA is a more robust approach to reflect a natural consumption effect of this compound rather than administrating it through intraperitoneal injection or oral gavage. From the ITT, we observed a significant increase in insulin resistance in mice fed a high-fat diet supplemented with CGA. We also assessed insulin stimulation in metabolic tissues by measuring the phosphorylation of Akt (Ser^{473}). Phosphorylation of Akt because of insulin stimulation is required for insulin-stimulated glucose uptake and anabolic metabolism.[32] Therefore, an increased rate of Akt (Ser^{473}) phosphorylation reflects insulin sensitivity in the tissue. We saw a tendency for reduced insulin-stimulated phosphorylation of Akt in the white adipose tissue of mice fed a high-fat diet supplemented with CGA, which reflects a mild insulin resistance. This result does not correspond to some *in vitro* studies showing improvement of insulin signaling in 3T3-L1 adipocytes by chicory salad leaf extract containing CGA.[33] However, our result supports the increased insulin resistance observed from the ITT. We did not observe an effect of CGA supplementation on insulin sensitivity in the liver and quadricep muscle, suggesting that insulin resistance did not occur in these tissues.

We also assessed effects of CGA supplementation on signaling pathways associated with fat oxidation in the liver, white adipose tissue, and skeletal muscle. We and others have highlighted that phosphorylation of ACCβ at its critical site (Ser^{79})[34] by AMPK leads to the decrease in activity of ACCβ, resulting in reduced malonyl-CoA production, which, in turn, relieves inhibition of carnitine palmitoyl transferase 1 (CPT-1) and increases fatty acid oxidation.[35] In our study, we saw a markedly reduced phosphorylation of AMPK (Thr^{172}) and its downstream target ACCβ (Ser^{79}) in the liver, suggesting that reduced fatty acid oxidation is occurring in this tissue. Haematoxylin and eosin staining of the liver tissue sections showed that CGA supplementation in the high-fat diet resulted in increased steatosis in the liver, which supports the impaired fatty acid oxidation signaling in this tissue. The CGA supplementation was also found to increase the lipid content in the liver by 1.5-fold when compared to the liver of mice fed a high-fat diet alone. Our data are not in agreement with the study by Ong et al.,[31] which found that CGA increases phosphorylation of AMPK and ACCβ in L6 myotubes after incubation with CGA *in vitro*. However, results from *in vitro* studies may differ from those found *in vivo*. In white adipose tissue, there was a tendency for reduced phosphorylation of ACCβ in mice fed a high-fat diet supplemented with CGA compared to mice fed a high-fat diet only, suggesting decreased fatty acid oxidation. Haematoxylin and eosin staining of the white adipose tissue sections revealed that CGA resulted in adipocyte hypertrophy in mice. This supports that the data showed a tendency for impaired fatty acid oxidation pathways in white adipose tissue. The result from our study is not in accordance with data shown in the study by Cho et al.,[26] where they demonstrated that CGA reduced obesity and improved lipid metabolism in obese ICR mice. However, the lower dose of CGA used may explain some of the variation in results compared to our study. We also assessed phosphorylation of AMPK and ACCβ in quadricep. However, we did not observe an effect of CGA on this tissue. Tsuda et al.[18] also did not find an effect of CGA on phosphorylation of these proteins in epitrochlearis muscle but observed that caffeic acid, a metabolite of CGA, induced the phosphorylation of AMPK and ACCβ.

Our study does not support the hypothesis that supplementation of CGA to a high-fat diet will protect against features of the metabolic syndrome in obese mice. Further work especially on human intervention studies is required to determine if coffee polyphenols are able to protect against metabolic syndrome and type 2 diabetes in humans.

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Notes

The authors declare no competing financial interest.

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