Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice

Takatoshi Murase, Koichi Misawa, Yoshihiko Minegishi, Masafumi Aoki, Hideo Ominami, Yasuto Suzuki, Yusuke Shibuuya, and Tadashi Hase

Biological Science Laboratories, Kao Corporation, Tochigi, Japan

Submitted 28 July 2010; accepted in final form 12 October 2010

Coffee polyphenols (CPP), which are abundant in coffee and consumed worldwide, on diet-induced body fat accumulation. C57BL/6J mice were fed either a control diet, a high-fat diet, or a high-fat diet supplemented with 0.5 to 1.0% CPP for 2–15 wk. Supplementation with CPP significantly reduced body weight gain, abdominal and liver fat accumulation, and infiltration of macrophages into adipose tissues. Energy expenditure evaluated by indirect calorimetry was significantly increased in CPP-fed mice. Similarly, CPP suppressed the expression of these molecules in Hepa 1–6 cells, concomitant with an increase in microRNA-122. Structure-activity relationship studies of nine quinic acid derivatives isolated from CPP in Hepa 1–6 cells suggested that mono- or di-caffeoyl quinic acids (CQA) are active molecules and stimulating energy expenditure in C57BL/6J mice. Here, we show that daily intake of CPP could be a potentially effective and safe approach to prevent or attenuate obesity and related diseases. Coffee is one of the most widely consumed beverages in the world. Recently, there has been increasing interest in the health benefits of coffee (8, 14). Several epidemiologic studies suggest that coffee consumption helps prevent chronic diseases, including type 2 diabetes (37, 46), liver diseases (17, 36), and Parkinson’s disease (35). These beneficial effects of coffee are at least partly attributed to caffeine, which is abundant in coffee. Coffee also contains many phenolic compounds [coffee polyphenols (CPP)] such as caffeoyl quinic acids (CQA; 5-CQA is also called chlorogenic acid) and feruloyl quinic acids (FQA). A 200-ml cup of roasted ground coffee supplies 20–675 mg CPP, and the daily intake of CPP by a coffee drinker is as much as 1 g (4).

We have been investigating the nutritional functions of plant polyphenols such as tea catechins (28, 30) and CPP (42, 43). We (42, 43) previously reported that 5-CQA and ferulic acid improve endothelial function and attenuate hypertension. In our studies of plant polyphenols, we evaluated the effects of various polyphenols on energy metabolism-related molecules and found that CPP suppresses high-fat diet-induced body fat accumulation by downregulating SREBP-1c and downstream molecules and stimulating energy expenditure in C57BL/6J mice. Here, we show that daily intake of CPP could be a potentially effective and safe approach to prevent or attenuate obesity and metabolic syndrome.

MATERIALS AND METHODS

Preparation of CPP

Roasted coffee beans were extracted with hot water, and the extract was reduced to a powder using the spray-dry method. The extract was dissolved in water and applied to an aromatic type adsorbent column.

Address for reprint requests and other correspondence: T. Murase, Biological Science Laboratories, Kao, 2606 Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497, Japan (e-mail: murase.takatoshi@kao.co.jp).
Purification and Isolation of Polyphenols from CPP

Nine types of quinic acid derivatives were isolated using a medium pressure chromatography system (Yamazen, Osaka, Japan) equipped with an Ultra Pack ODS-A-40D column, UV detector PREP-UV-10V, fraction collector FR 50N, gradient mixer GR200, degassing unit, and pump PUMP-600A. CPP (2.0 g) dissolved in 20 ml solution A (acetic acid-methanol-water = 1:20:80) was applied to the ODS column and eluted with solution A and solution B (methanol) at a flow rate of 10 ml/min as follows: 0–100 min, solution A (100%); 100–600 min, solution B (0%–100%). 3-CQA; 3,4-diCQA; 3,5-diCQA; and 4,5-diCQA were isolated as single compounds in this process. Both the CPP powder contained no caffeine. The chemical structures of 5-CQA; 3,5-di-CQA; and 5-FQA are shown in Fig. 1.

Animals and Diets

Experiment 1. Male 7-wk-old C57BL/6J mice obtained from Charles River (Kanagawa, Japan) were maintained at 23 ± 2°C under a 12-h light-dark cycle (lights on from 0700–1900). The mice were divided into four groups (n = 10, 5 mice/cage) and allowed ad libitum access to water and one of the following synthetic diets: a control diet containing 5% (wt/vol) and spray-dried, and CPP was obtained. The CPP composition was measured by HPLC. Total polyphenol content of the CPP was 77.1%. The composition of polyphenols was 7.7% 3-CQA; 15.5% 4-CQA; 31.9% 5-CQA; 7.6% 3-FQA; 5.6% 4-FQA; 8.5% 5-FQA; 9.0% 3,4-di-CQA; 8.9% 3,5-di-CQA; and 5.3% 4,5-di-CQA. The CPP powder containing 5% (wt/wt) fat, 20% casein, 66.5% potato starch, 4% cellulose, 3.5% vitamins, and 1% minerals; a high-fat diet containing 30% fat, 20% casein, 28.5% starch, 13% sucrose, 4% cellulose, 3.5% vitamins, and 1% minerals; or a CPP diet, which comprised the high-fat diet supplemented with the indicated amount of CPP. Animals were maintained on these diets for 15 wk. Food intake was measured every 2–3 days on a per-cage basis throughout the study using two Roden CAFE (Oriental Yeast, Tokyo, Japan) food dispensers per cage to minimize dispersion of the diet.

Experiment 2. Mice were maintained on the respective diets (n = 8), and feces were collected during the fifth week for measurement of the fecal lipid content. At 9 wk, the metabolic rate was determined by indirect calorimetry as described below.

Experiment 3. Mice (n = 8–10) were allowed ad libitum access to the respective synthetic diets for 2 wk. On the final day of the experiment, various tissues were dissected from each animal and frozen in liquid nitrogen for subsequent RNA isolation and measurement of ACC activity and malonyl-CoA levels.

All animal experiments were approved by the Animal Care Committee of Kao Tochigi Institute. All animal experiments followed the Committee Guidelines for the Care and Use of Laboratory Animals.

Extraction of Total Fat from the Feces

Feces were collected three times on a per-cage basis over a 24-h period during the fifth week of experiment 2. After freeze drying, the total lipid content of the feces was extracted according to the procedure of Jeejeebhoy et al. (18), and the amount of extracted lipid was determined gravimetrically.

Oil Red O Staining

Oil Red O staining was performed following the procedure described previously (33), with minor modifications. In brief, frozen sections of livers fixed in phosphate-buffered 10% formaldehyde were stained with a 0.35% solution of Oil Red O in isopropanol alcohol for 15 min. After being washed with water, the sections were stained with hematoxylin and photographed.

Hepatic Lipid Analysis

On the final day of experiment 1, the livers were dissected from each animal. Total lipid was extracted from the liver using Folch’s method (11). The extracts were dried under nitrogen gas, resolved in 2-propanol containing 10% Triton X-100, and subjected to lipid analyses. Triglyceride and total cholesterol concentrations were assayed using the Triglyceride E-test Wako or Cholesterol E-test Wako kit (Wako, Osaka, Japan), respectively.

Detection of Macrophages in Adipose Tissue

Epididymal adipose tissue samples were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sections (3 μm) were mounted on slides, deparaffinized in xylene, and stained for F4/80 with anti-F4/80 primary antibody (Serotec, Oxford, UK) and biotinylated-anti-rat IgG (DAKO, Tokyo, Japan) as a secondary antibody, using standard immunohistochemistry methods (47). After incubation with peroxidase-labeled streptavidin, the peroxidase substrate diaminobenzidine was added and the sections were further incubated. Sections were rinsed and counterstained with Mayer’s hematoxylin. Mounting solution and coverslips were added, and the sections were photographed.

Blood Analysis

Blood was collected after 3 h of fasting by cutting the tail 1 wk before the end of experiment 1. Plasma triglyceride, total cholesterol, nonesterified fatty acid, glucose, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, insulin, and leptin levels were determined using commercially available kits.
Cell Culture

Hepa 1–6 (mouse hepatoma) cells were purchased from Dainippon Sumitomo Pharma (Osaka, Japan) and maintained in DMEM supplemented with 10% FBS and 10 ml/l antibiotic-antimycotic mixture (GIBCO, Grand Island, NY) in an atmosphere of 95% air-5% CO2 at 37°C. After the cells reached subconfluence, they were cultured in serum-free medium for an additional 12 h and then exposed to the indicated concentrations of various compounds for 24 h.

RNA Extraction and RT-PCR

For real-time RT-PCR analyses, total RNA was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) and amplified on a thermal cycler (7500 Fast Real-Time PCR system; Applied Biosystems, Foster City, CA) using a Power Sybr Green Master Mix (Applied Biosystems; Ref. 27). The primers used were as follows: 36B4-F: CGTACTCCAGAGGATTGT; 36B4-R: CAGGAGGAGCGCTGTTAC; PPARα: TGAGGAATCATACAGACCAGG; PPARγ: CTTGGCTACACATACCGG; ACC2: GATGACCTCTGGATGTTCTTG; ACO: CAGGGGCTTCAAGTGCTTG; ACC1: GGAGATGTACGCTGACCGAG; COX-I: TCATACCTCAAAGCAACGAAGC; COX-I-R: GGGCGGGATCAGCCAGAA; ACO-R: CAGGGGCTTCAAGTGCTTG; PPARγ/H9251, rat PPARγ/LBD, and PPARγ/H9252, rat PPARγ/LBD chimeric receptor expression plasmids (Thermo Scientific, Rockford, IL). Western blotting was performed using anti-SREBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and specific antibodies (Cell Signaling, Beverly, MA; Ref. 29). For PPAR Luciferase Assay, the pG5luc reporter plasmid containing the GAL4 binding site was inserted into the pBIND-GAL4 expression plasmid (Promega, Madison, WI).

RT-PCR for Measurement of microRNA-122

Twenty-four hours after Hepa 1–6 cells were treated with each sample, microRNA-122 (miRNA) was purified using an miNeasy mini (Qiagen). Reverse transcription and PCR were performed using an miScript reverse transcription kit (Qiagen), miScript SYBR Green PCR kit (Qiagen), and an miScript primer assay (Qiagen) according to the manufacturer’s instructions. miR-122 expression levels were normalized to RNA U6.

Measurement of ACC Activity

ACC activity was measured according to the methods of Tanabe et al. (45) with minor modifications. Briefly, livers or cells were homogenized in ice-cold 250 mM sucrose solution containing protease inhibitors using a Dounce homogenizer or by passing them through a 25-gauge needle. The homogenates were centrifuged at 100,000 g for 30 min at 4°C, followed by gel filtration on a Sephadex G-50, and the protein content was determined. ACC activity was assayed with an assay mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM potassium citrate, 10 mM MgCl2, 0.125 mM acetyl-CoA, 0.5 mM phosphoenolpyruvate, 0.125 mM NADH, 15 μg/ml pyruvate kinase, 6 μg/ml lactate dehydrogenase, 3.75 mM glutathione, 0.75 mg/ml BSA, 3.75 mM ATP, and 25 mM KHC03.

Measurement of Malonyl-CoA

Malonyl-CoA content was measured as previously described using reversed-phase HPLC (6). The livers were homogenized in ice-cold (5°C) homogenization solution (5% sulfo salicylic acid and 50 μM dithiothreitol) using a teflon-coated homogenizer (Microtech, Chiba, Japan) in a microcentrifuge tube. The homogenates were then centrifuged at 15,000 g for 10 min at 4°C to obtain the supernatant.

Hepa 1–6 cells treated with or without test samples for 24 h were harvested and sonicated in homogenization solution at 4°C. Homogenates were centrifuged at 15,000 g for 10 min, and the upper layer was applied to ODS Hypersil (C18) column chromatography (Phenomenex, Torrance, CA). Elution solvent A comprised 100 mM sodium phosphate and 75 mM sodium acetate adjusted to pH 4.6. Solvent B comprised 70% solvent A in methanol. The profile of the gradient was as follows: 0 min, 90% solvent A; 10 min, 60% solvent A; and 17.6 min, 10% solvent A. HPLC chromatographic peaks were identified using a 254-nm UV detector (L-4250 UV-VIS detector; Hitachi, Japan).

Western Blot Analyses of Phospho-AMPKα, Phospho-ACC, and SREBP-1

Hepa 1–6 cells treated with CPP or 5-CQA were washed with ice-cold PBS and lysed in 200 μl lysis buffer [10 mM Tris-HCl (pH 7.4), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 0.5% Triton X-100, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail-1 and -2 (Sigma)]. The lysates were homogenized by passage through a 25-G needle and kept on ice for 30 min. After centrifugation at 16,000 g for 15 min at 4°C, the supernatants were collected and their protein content was determined. Equal amounts of protein were separated by SDS-PAGE, and Western blotting was performed using anti-phospho-AMPKα- or anti-phospho-ACC-specific antibodies (Cell Signaling, Beverley, MA; Ref. 29). For SREBP-1 analysis, nuclear extracts of treated Hepa 1–6 cells were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific, Rockford, IL). Western blotting was performed using anti-SREBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-histone H1 antibody (Santa Cruz Biotechnology). After being washed with 0.1% Tween-20/BS, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin as a secondary antibody. Bands were visualized with chemiluminescence reagent (LumiGLO; Cell Signaling) and a Chemiluminescence reagent (LumiGLO; Cell Signaling) and a Chemiluminescence reagent (LumiGLO; Cell Signaling) and a Chemiluminescence reagent (LumiGLO; Cell Signaling) and a Chemiluminescence reagent (LumiGLO; Cell Signaling) and a Chemiluminescence reagent (LumiGLO; Cell Signaling).
cells were then incubated for 4 h in fresh DMEM (+5% charcoal-treated FBS). After treatment with or without each sample (CPP: 0.0025%; CQA/FQAs/diCQA: 50 μM; Wy14643: 10 μM; pioglitazone: 10 μM; and GW501516: 10 nM) for 20 h, cells were lysed, and then firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Wy14643 (Sigma) was used as a positive control for PPARα, pioglitazone (Wako) for PPARγ, and GW501516 (Wako) for PPARδ.

**Indirect Calorimetry**

Energy metabolism studies were performed during the ninth week of experiment 2 using a magnetic-type mass spectrometric calorimeter ARCO-2000 (ARCO System, Chiba, Japan). Each mouse was placed in a chamber for 10–12 h and allowed to acclimatize to the surroundings before the measurements. Oxygen consumption and carbon dioxide production were then measured under feeding conditions (free access to the control diet) for 24 h. The respiratory quotient (RQ) was calculated from the measured values of oxygen consumption (V\(\dot{O}_2\)) and carbon dioxide exhalation (V\(\dot{CO}_2\)). Locomotor activity was measured using an automated motion analysis system (Actracer-2000; ARCO System), which detects the amount of centroid fluctuation using a weighted transducer.

**Statistical Analysis**

All values are presented as the means ± SE. Statistical analysis was conducted using unpaired t-tests or ANOVA followed by Dunnett’s test (StatView; SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant. Comparisons of the temporal body weight changes between groups were conducted by two-way ANOVA.

**RESULTS**

**CPP Decreases Body Fat Accumulation**

Consistent with previous reports (30, 41), feeding C57BL/6J mice a high-fat diet significantly increased body weight compared with a control diet (\(P < 0.001\), two-way ANOVA). The addition of CPP to the high-fat diet dose dependently reduced body weight gain (Fig. 2A; \(P < 0.001\), two-way ANOVA). Cumulative energy intake did not significantly differ among the high-fat control group and the CPP groups (Fig. 2B). Supplementation with 1.0% CPP almost abolished the high-fat diet-induced epididymal, mesenteric, retroperitoneal, and perirenal fat accumulation in parallel with the body weight reduction (Table 1). Fecal excretion of lipids was greater in the high-fat group than the control diet group. Fecal excretion of lipids did not differ between the high-fat control and CPP groups, suggesting that CPP does not affect the absorption rate of dietary fat under our experimental conditions.

**CPP Attenuates Hepatic Lipid Accumulation**

CPP induced significant changes in the liver weight and lipid contents. The high-fat diet increased liver weight by 18%, and the increase was significantly suppressed in a dose-dependent manner by CPP ingestion (Table 1). Oil Red O staining of the liver tissue sections showed that CPP ingestion reduced high-fat diet-induced lipid accumulation in the liver (Fig. 2C). Quantitative analysis of the liver lipid contents revealed that the intake of 1.0% CPP suppressed the high-fat diet-induced increase in the liver triglyceride and cholesterol content by 79 and 90%, respectively (Fig. 2, D and E).

**CPP Decreases Macrophage Infiltration into Adipose Tissue**

CPP-fed mice had smaller adipocytes than high-fat control mice (Fig. 3A). Increased adiposity is associated with increased infiltration of macrophages into the adipose tissue (47). Therefore, we determined the effect of CPP on inflammatory responses in epididymal adipose tissue. Consistent with previous reports, staining of adipose tissue with antibodies to the macrophage marker F4/80 showed a high frequency of macrophages in the adipose tissue of high-fat diet-fed mice, which was confirmed by gene expression analysis of F4/80 and CD68 with real-time PCR (Fig. 3B). In the adipose tissue of mice fed CPP, however, the number of macrophages was decreased compared with a control diet (\(P < 0.05\); Table 1). Oil Red O staining of the liver tissue sections showed that CPP ingestion reduced high-fat diet-induced lipid accumulation in the liver (Fig. 2C). Quantitative analysis of the liver lipid contents revealed that the intake of 1.0% CPP suppressed the high-fat diet-induced increase in the liver triglyceride and cholesterol content by 79 and 90%, respectively (Fig. 2, D and E).

**CPP Attenuates Hepatic Lipid Accumulation**

Consistent with previous reports (30, 41), feeding C57BL/6J mice a high-fat diet significantly increased body weight compared with a control diet (\(P < 0.001\), two-way ANOVA). The addition of CPP to the high-fat diet dose dependently reduced body weight gain (Fig. 2A; \(P < 0.001\), two-way ANOVA). Cumulative energy intake did not significantly differ among the high-fat control group and the CPP groups (Fig. 2B). Supplementation with 1.0% CPP almost abolished the high-fat diet-induced epididymal, mesenteric, retroperitoneal, and perirenal fat accumulation in parallel with the body weight reduction (Table 1). Fecal excretion of lipids was greater in the high-fat group than the control diet group. Fecal excretion of lipids did not differ between the high-fat control and CPP groups, suggesting that CPP does not affect the absorption rate of dietary fat under our experimental conditions.

**CPP Decreases Macrophage Infiltration into Adipose Tissue**

CPP-fed mice had smaller adipocytes than high-fat control mice (Fig. 3A). Increased adiposity is associated with increased infiltration of macrophages into the adipose tissue (47). Therefore, we determined the effect of CPP on inflammatory responses in epididymal adipose tissue. Consistent with previous reports, staining of adipose tissue with antibodies to the macrophage marker F4/80 showed a high frequency of macrophages in the adipose tissue of high-fat diet-fed mice, which was confirmed by gene expression analysis of F4/80 and CD68 with real-time PCR (Fig. 3B). In the adipose tissue of mice fed CPP, however, the number of macrophages was decreased compared with a control diet (\(P < 0.05\); Table 1). Oil Red O staining of the liver tissue sections showed that CPP ingestion reduced high-fat diet-induced lipid accumulation in the liver (Fig. 2C). Quantitative analysis of the liver lipid contents revealed that the intake of 1.0% CPP suppressed the high-fat diet-induced increase in the liver triglyceride and cholesterol content by 79 and 90%, respectively (Fig. 2, D and E).
concomitant with decreased expression of F4/80 and CD68 mRNA. Consistent with the increased macrophage content, the expression of monocyte chemoattractant protein-1, a chemokine involved in macrophage recruitment, was upregulated nearly fourfold by the high-fat diet. CPP decreased the high-fat diet-induced increase in monocyte chemoattractant protein-1.

CPP Enhances Whole Body Metabolic Rate

Whole-body energy metabolism was determined by monitoring oxygen consumption (energy expenditure) and RQ by indirect calorimetry. Oxygen consumption was greater in the CPP group than in the high-fat control group throughout the monitoring period, although RQ values did not differ between groups (Fig. 4, A and B). Locomotor activity did not significantly differ between the high-fat control and CPP groups (Fig. 4C).

CPP Decreases mRNA Expression of Lipogenic Enzymes

To elucidate the underlying mechanisms of the effects of CPP, we used quantitative RT-PCR after 2 wk of feeding, during which there were no significant differences in food intake or body weight among the high-fat groups, to examine CPP-induced early changes in gene expression in various tissues (experiment 3). Supplementation with CPP significantly downregulated the mRNA levels of FAS, ACC1, and SCD1, which are lipogenic enzymes in the liver, compared with the

Table 1. Effects of CPP on body fat accumulation and serum components in C57BL/6J mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight, g</td>
<td>35.8±0.6†</td>
<td>42.8±1.1</td>
<td>39.2±1.3</td>
<td>36.7±1.3†</td>
</tr>
<tr>
<td>Perirenal fat, g</td>
<td>0.17±0.02*</td>
<td>0.33±0.04</td>
<td>0.24±0.04</td>
<td>0.15±0.03†</td>
</tr>
<tr>
<td>Epididymal fat, g</td>
<td>1.38±0.14†</td>
<td>2.23±0.11</td>
<td>2.08±0.16</td>
<td>1.49±0.20†</td>
</tr>
<tr>
<td>Mesenteric fat, g</td>
<td>0.61±0.05†</td>
<td>1.05±0.14</td>
<td>0.76±0.09</td>
<td>0.58±0.11†</td>
</tr>
<tr>
<td>Retroperitoneal fat, g</td>
<td>0.36±0.03†</td>
<td>0.56±0.02</td>
<td>0.52±0.03</td>
<td>0.38±0.05†</td>
</tr>
<tr>
<td>Liver, g</td>
<td>1.41±0.04*</td>
<td>1.67±0.08</td>
<td>1.50±0.08</td>
<td>1.40±0.06*</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td>0.0108</td>
<td>0.0151</td>
<td>0.0120</td>
<td>0.0109</td>
</tr>
<tr>
<td>Lipids in feces, mg/g</td>
<td>75.7±2.5†</td>
<td>181.5±5.3</td>
<td>NT</td>
<td>180.2±4.1</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>23.0±3.8†</td>
<td>57.6±7.7</td>
<td>44.7±6.2</td>
<td>23.6±8.2†</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.94±0.23*</td>
<td>3.44±0.51</td>
<td>2.04±0.31*</td>
<td>1.89±0.34*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>230.3±9.0†</td>
<td>296.5±9.6</td>
<td>270.0±9.1</td>
<td>258.9±10.1*</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>152.3±6.9</td>
<td>170.6±5.6</td>
<td>160.5±5.0</td>
<td>144.9±4.7†</td>
</tr>
<tr>
<td>NEFA, mEq/l</td>
<td>152.3±6.9</td>
<td>230.3±9.0</td>
<td>170.6±5.6</td>
<td>160.5±5.0</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>41.4±2.7</td>
<td>40.6±1.7</td>
<td>40.0±1.0</td>
<td>40.0±1.0</td>
</tr>
<tr>
<td>GOT, U/l</td>
<td>18.8±1.5</td>
<td>19.2±2.9</td>
<td>16.2±1.0</td>
<td>17.4±1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 mice. Feed efficiency was calculated as follows: [body weight gain per cage (g)/kcal of food consumed per cage], CPP, coffee polyphenols; NEFA, nonesterified fatty acid; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; NT, not tested. *P<0.05, †P<0.01 vs. the high-fat (HF) group by Dunnett’s test.

Fig. 3. Infiltration of macrophages into adipose tissues. A: representative F4/80 immunostaining of macrophages in epididymal adipose tissues from mice fed each diet for 15 wk (experiment 1). B: total RNA isolated from epididymal adipose tissues was subjected to real-time RT-PCR. Amount of each mRNA was normalized to 36B4 mRNA levels and is expressed as a percentage of the corresponding amount in the high-fat control group. Values are means ± SE of 10 mice. *P<0.05, **P<0.01 vs. high-fat control group (Dunnett’s test). MCP-1, monocyte chemoattractant protein-1.
levels in the high-fat control group (Fig. 5A). The mRNA level of SREBP-1c, the master regulator of fatty acid synthesis (10, 39), was lower in the CPP group than in the high-fat control group. The mRNA levels of ACC2, another ACC isozyme, were also decreased by CPP feeding. The mRNA level of PDK4, a key regulatory enzyme involved in switching the energy source from glucose to fatty acids and glucose homeostasis by phosphorylating the pyruvate complex (40), was also significantly decreased by CPP. On the other hand, there were no marked differences in the mRNA levels of ACO, PPARγ, MGAT-1, or DGAT-2 between the groups.

Similarly, CPP ingestion decreased mRNA levels of lipogenic enzymes, including FAS, SCD1, ACC1, and SREBP-1c, in perirenal white adipose tissue (Fig. 5B). UCP-2 mRNA was increased by CPP. Although ACC2 in brown adipose tissue was significantly lower in the CPP group, no marked changes were observed in other molecules in the brown adipose tissue or in the gastrocnemius muscle (Fig. 5, B and C). These findings indicate that CPP downregulates the expression of the genes involved in fatty acid synthesis and energy metabolism and that the effects of CPP intake are more marked in the liver and white adipose tissue, suggesting that these organs are the primary targets of CPP and appear to make important contributions to the effects of CPP.

CPP Decreases ACC Activity and Malonyl-CoA Levels in the Liver of Mice

In accordance with the changes in mRNA expression, ingestion of CPP decreased ACC activity in the liver of mice (Fig. 6A). In addition, levels of malonyl-CoA, which is generated by ACC from acetyl-CoA and negatively regulates CPT activity, were significantly lower in CPP-fed mice than control mice (Fig. 6B). These results suggest that CPP activates fatty acid catabolism through an ACC-malonyl-CoA-mediated pathway.

CPP Suppresses the Lipogenic Pathway In Vitro

To further clarify the effects of CPP, we examined the influence of CPP and its constituents on gene expression in Hepa 1–6 cells. CPP significantly suppressed the expression of FAS, ACC1, ACC2, SREBP-1c, and PDK4 mRNA (Fig. 7A). CPP is composed of nine types of quinic acid derivatives (3 CQAs, 3 di-CQAs, and 3 FQAs); therefore, to further characterize the molecular specificity of quinic acid derivatives, we examined the effects of the nine quinic acid derivatives isolated from CPP. Treatment of cells with 5 μM of the quinic acid derivatives (3-, 4-, 5-CQA; or 3,4-, 3,5-, 4,5-di-CQA), all of which have one or two caffeic acid moieties, suppressed the expression of FAS, ACC1, ACC2, and SREBP-1c mRNA, and...
to a lesser extent PDK4, whereas 5 µM FQA had no significant effect (Fig. 7B). Similar to the results of mRNA analyses, CPP or 5-CQA treatment decreased the amount of the nuclear active form of SREBP-1 protein (Fig. 8A). CPP or 5-CQA treatment also decreased ACC activity, consistent with their mRNA levels (Fig. 8B). In conjunction with decreased ACC mRNA and activity levels, CPP or 5-CQA treatment also significantly decreased malonyl-CoA levels (Fig. 8C), suggesting that CPP stimulates lipid metabolism by downregulating ACC2 and lowering malonyl-CoA levels.

CPP Increases miR-122 Levels in Hepa 1–6 cells

MicroRNA-122 (miR-122) is abundantly expressed in hepatocytes and plays an important role in regulating lipid metabolism (2, 9, 12, 16). Treatment of Hepa 1–6 cells with CPP or 5-CQA significantly increased miR-122 by 35 and 26%, respectively (Fig. 8D), suggesting a potential role for miR-122 in the effects of CPP.

CPP Does Not Induce Phosphorylation of AMPKα and ACC in Hepa 1–6 Cells

AMPK functions as an energy sensor that regulates metabolic pathways in fatty acid and glucose metabolism (23). To reveal the effects of CPP on the AMPK pathway, phosphorylation of AMPKα and the downstream target ACC were examined by Western blotting. Treatment of Hepa 1–6 cells with 5-aminomidazole-4-carboxamide riboside, an AMPK activa-
tor, increased phosphorylated AMPK and ACC; however, CPP and 5-CQA had no effect (Fig. 9A).

**CPP Does Not Act as a Ligand for PPARs**

We tested whether CPP and 5-CQA act as PPAR ligands using the luciferase assay system. Although Wy14643 (a PPARα agonist), GW501516 (a PPARβ agonist), and pioglitazone (a PPARγ agonist) significantly enhanced PPAR-dependent luciferase activities, CPP and its constituents (CQAs, di-CQAs, and FQAs) had no marked effects, indicating that CPP does not act as direct ligand for PPAR (Fig. 9B).

**DISCUSSION**

The results of the present study demonstrated that long-term intake of CPP significantly suppressed high-fat diet-induced visceral and liver fat accumulation, attenuated the increase in plasma glucose and insulin concentrations, and concomitantly increased energy expenditure. Histologic analysis of the adipose tissue revealed that CPP-fed mice had smaller adipocytes than high fat-fed mice without CPP, which correlated with a lower number of infiltrating macrophages. Obesity-associated low-grade inflammation characterized by an increased abundance of macrophages in the adipose tissue is recognized as a key step in the pathogenesis of insulin resistance (32). Therefore, CPP may be useful as a potential countermeasure against obesity and type 2 diabetes.

Many of the beneficial effects of coffee are considered to be mediated by caffeine (8, 14, 17, 35, 36, 37, 46). Because CPP does not contain caffeine, however, the effects of CPP observed in the present study were due to the effects of the polyphenols and not caffeine. There was no marked difference in the total energy intake between high-fat control and CPP groups, and CPP did not increase the fecal excretion of lipids; therefore, various mechanisms leading to increased energy expenditure or lower fat deposition might be involved in the effects of CPP.

CPP downregulated lipogenic enzymes, such as FAS, ACC1, and SCD1, in the liver and adipose tissue. Several lines of evidence indicate that the suppression or disruption of ACC1, a cytosolic enzyme that catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, leads to the reduction of hepatic triglyceride synthesis and accumulation (24, 38), suggesting that suppression of ACC1 expression could contribute to reduce body fat accumulation. SCD1, which catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids, also has an important role in energy metabolism and body weight regulation (7). Mice with targeted disruption of the SCD1 gene have increased energy expenditure, reduced adiposity, and are resistant to diet-induced obesity (31). Treatment of mice with SCD1-specific antisense oligonucleotide inhibitors results in a higher metabolic rate, prevention of diet-induced obesity, and steatosis (19); therefore, decreased SCD1 expression could explain the increased energy expenditure and subsequent body fat reduction observed in CPP-fed mice. The expression of several lipogenic genes is regulated by SREBP-1c at the transcriptional level (10, 39). CPP intake decreased SREBP-1c mRNA levels in both the liver and adipose tissue of mice. In addition, CPP decreased the expression of lipogenic genes along with a decrease in SREBP-1c mRNA and the mature form of SREBP-1 protein in Hepa 1–6 cells. Experiments in cultured cells may not necessarily mimic the condition of high-fat diet-fed mice. Results in cultured cells, however, are largely consistent with those observed in mice. Further, in our preliminary studies, CPP also reduced SREBP-1c and FAS mRNA expression in the liver under the control diet condition (data not shown). Overall, the results suggest that CPP suppresses the lipogenic pathway by down-regulating SREBP-1c, which leads to a reduced accumulation of body fat (Fig. 10).
miR-122, a miRNA that comprises ~70% of all miRNA in the liver, is proposed to control lipogenic gene expression by interfering with the translation or stability of target mRNA (2, 9, 12, 16). Cheung et al. (2) recently reported that miR-122 levels were significantly decreased in subjects with nonalcoholic steatohepatitis and that overexpression of miR-122 in HepG2 cells led to a significant decrease in mRNA and protein levels of SREBP-1c. Treatment of Hepa 1–6 cells with CPP or 5-CQA significantly increased miR-122 levels, concomitant with a decrease in the levels of SREBP-1c mRNA and protein, suggesting a possible role of miR-122 in the regulation of the expression of SREBP-1c and the downstream targets FAS and ACC by CPP in hepatocytes. It is important to note, however, that in contrast to the report by Cheung et al. (2), others (9, 16) have reported an opposite effect of miR-122 on lipogenic gene expression. In addition, as is the case with another miRNA, little is known about the intermediate steps that lead to the up- or downregulation of miR-122 and the regulation of downstream target gene expression by miR-122. Therefore, further studies are necessary to clarify the detailed mechanisms of miR-122-mediated regulation of SREBP-1c and its related molecules.

SREBP-1c expression is transcriptionally regulated by liver X receptor-α (LXRα; Ref. 34). Moreover, the LXRA-SREBP-1c pathway is negatively regulated by AMPK, a key regulatory enzyme that acts to promote ATP-generating pathways (23), directly through threonine phosphorylation of LXRα and indirectly through the inhibition of the mammalian target of the rapamycin-p70 ribosomal S6 kinase-1 pathway (15). CPP and 5-CQA, however, did not induce AMPKα phosphorylation in cultured cells, suggesting that the effects of CPP are not directly mediated through AMPK-dependent pathways.

miR-122, a miRNA that comprises ~70% of all miRNA in the liver, is proposed to control lipogenic gene expression by interfering with the translation or stability of target mRNA (2, 9, 12, 16). Cheung et al. (2) recently reported that miR-122 levels were significantly decreased in subjects with nonalcoholic steatohepatitis and that overexpression of miR-122 in HepG2 cells led to a significant decrease in mRNA and protein levels of SREBP-1c. Treatment of Hepa 1–6 cells with CPP or 5-CQA significantly increased miR-122 levels, concomitant with a decrease in the levels of SREBP-1c mRNA and protein, suggesting a possible role of miR-122 in the regulation of the expression of SREBP-1c and the downstream targets FAS and ACC by CPP in hepatocytes. It is important to note, however, that in contrast to the report by Cheung et al. (2), others (9, 16) have reported an opposite effect of miR-122 on lipogenic gene expression. In addition, as is the case with another miRNA, little is known about the intermediate steps that lead to the up- or downregulation of miR-122 and the regulation of downstream target gene expression by miR-122. Therefore, further studies are necessary to clarify the detailed mechanisms of miR-122-mediated regulation of SREBP-1c and its related molecules.

SREBP-1c expression is transcriptionally regulated by liver X receptor-α (LXRα; Ref. 34). Moreover, the LXRA-SREBP-1c pathway is negatively regulated by AMPK, a key regulatory enzyme that acts to promote ATP-generating pathways (23), directly through threonine phosphorylation of LXRα and indirectly through the inhibition of the mammalian target of the rapamycin-p70 ribosomal S6 kinase-1 pathway (15). CPP and 5-CQA, however, did not induce AMPKα phosphorylation in cultured cells, suggesting that the effects of CPP are not directly mediated through AMPK-dependent pathways.

miR-122, a miRNA that comprises ~70% of all miRNA in the liver, is proposed to control lipogenic gene expression by interfering with the translation or stability of target mRNA (2, 9, 12, 16). Cheung et al. (2) recently reported that miR-122 levels were significantly decreased in subjects with nonalcoholic steatohepatitis and that overexpression of miR-122 in HepG2 cells led to a significant decrease in mRNA and protein levels of SREBP-1c. Treatment of Hepa 1–6 cells with CPP or 5-CQA significantly increased miR-122 levels, concomitant with a decrease in the levels of SREBP-1c mRNA and protein, suggesting a possible role of miR-122 in the regulation of the expression of SREBP-1c and the downstream targets FAS and ACC by CPP in hepatocytes. It is important to note, however, that in contrast to the report by Cheung et al. (2), others (9, 16) have reported an opposite effect of miR-122 on lipogenic gene expression. In addition, as is the case with another miRNA, little is known about the intermediate steps that lead to the up- or downregulation of miR-122 and the regulation of downstream target gene expression by miR-122. Therefore, further studies are necessary to clarify the detailed mechanisms of miR-122-mediated regulation of SREBP-1c and its related molecules.

SREBP-1c expression is transcriptionally regulated by liver X receptor-α (LXRα; Ref. 34). Moreover, the LXRA-SREBP-1c pathway is negatively regulated by AMPK, a key regulatory enzyme that acts to promote ATP-generating pathways (23), directly through threonine phosphorylation of LXRα and indirectly through the inhibition of the mammalian target of the rapamycin-p70 ribosomal S6 kinase-1 pathway (15). CPP and 5-CQA, however, did not induce AMPKα phosphorylation in cultured cells, suggesting that the effects of CPP are not directly mediated through AMPK-dependent pathways.
The CPP-induced increase in the metabolic rate and subsequent reduction of body fat accumulation might also be mediated by decreased malonyl-CoA due to reduced ACC2 expression. ACC2 is primarily expressed in oxidative tissues, such as the heart, skeletal muscle, and liver, and malonyl-CoA formed at the mitochondrial surface by ACC2 acts to regulate mitochondrial fatty acid oxidation through allosteric inhibition of CPT-1 (25). A growing body of evidence from studies of ACC2 null mice (3), ACC inhibitors (38), and AMPK (23) indicates that the suppression of ACC2 beneficially affects energy metabolism and obesity, i.e., suppression of ACC2 increases energy expenditure, reduces hepatic and body fat mass, and reduces plasma glucose levels. These previous observations together with the present findings indicate that the suppression of ACC2 expression by CPP might lead to the loss of CPT-1 inhibition by decreasing the concentration of malonyl-CoA, which results in increased fatty acid oxidation and contributes to reduce body fat accumulation. Knocking out ACC2 was expected to mainly promote fatty acid oxidation, but Choi et al. (3) recently reported that both fat and carbohydrate oxidation are simultaneously increased in ACC2-knockout mice, resulting in an increase in total energy expenditure. These observations are similar to those observed in CPP-fed mice (increased total energy expenditure and constant RQ), suggesting that the effects of CPP are partly due to the suppression of ACC2.

An alternative explanation for the constant RQ value in CPP-fed mice despite the decrease in malonyl-CoA and predicted increase in fatty acid oxidation is that PDK4 modulates the balance between glucose and fatty acid utilization. PDK4 is a key regulatory enzyme involved in switching the energy source from glucose to fatty acids in response to physiologic conditions (40). CPP decreased levels of PDK4 mRNA in the liver and Hepa 1–6 cells. A decrease in PDK4 might facilitate the activity of the pyruvate dehydrogenase complex, which catalyzes the decarboxylation of pyruvate to acetyl-CoA, linking glycolysis to the carboxylic acid cycle, thereby favoring glucose oxidation. These results suggest that CPP facilitates not only fatty acid oxidation by decreasing ACC2 and malonyl-CoA levels but also glucose oxidation by downregulating PDK4, thus modulating the balance between glucose and fatty acid utilization.

Studies on the molecular specificity of the nine types of quinic acid derivatives in the regulation of ACC1, ACC2, SREBP-1c, and PDK4 mRNA expression in cultured cells revealed that CQAs or di-CQAs downregulate these enzymes (Fig. 7B), suggesting that these compounds largely contribute to the effects of CPP.

PPARs also have important roles in the regulation of energy metabolism (22). CPP and its constituents (CQAs, di-CQAs, and FQAs), however, did not affect PPAR-dependent reporter activity in a transient transfection assay, suggesting that the effects of CPP are not directly mediated through PPAR-dependent pathways.

Little is known about the recognition and transport mechanism of CPP. In recent years, a cell surface 67-kDa laminin receptor (67LR) was identified as a specific receptor for epigallocatechin gallate (44), a main constituent of green tea polyphenol, and its physiological significance has been investigated. The finding provides the possibility that CPP also exerts its biological actions through specific receptor-mediated signaling pathways. However, at present, the receptor and transport system for CPP remain unidentified; therefore, further studies are needed to better understand the entire picture of the action mechanism of CPP.

The findings of the present study suggest that sufficient intake of CPP might prevent or reduce body fat accumulation by modulating fatty acid or glucose metabolism and could potentially reduce the risk of obesity-associated diseases, including type 2 diabetes. Plasma CPP concentration after drinking coffee in humans is reported to reach 5–12 μM (26); therefore, CPP could also have these effects on body fat.

![Proposed mechanism for the CPP-mediated regulation of energy metabolism in hepatic cells.](http://ajpendo.physiology.org/)

---

**Fig. 10. Proposed mechanism for the CPP-mediated regulation of energy metabolism in hepatic cells.** CPP decreases ACC1, FAS, SCD1, and ACC2 by downregulating the upstream transcription factor SREBP-1c. Expression of SREBP-1c might be regulated by miR-122. Decrease in ACC2 leads to a decrease in the cellular malonyl-CoA content, thereby activating carnitine palmitoyl transferase-1 (CPT-1) and increasing the transport of fatty acids into the mitochondria for β-oxidation. CPP-induced reduction of PDK4 leads to increased utilization of glucose, thereby balancing whole body energy metabolism between glucose and fatty acids. Reduced lipogenesis and increased energy expenditure result in the decreased accumulation of body fat.
accumulation in humans. Coffee is one of the most widely consumed beverages in the world, and daily intake of CPP by coffee drinkers amounts to 1 g (4). Thus CPP has the benefit of long intake experience and may have an advantage over other similar strategies in safely counteracting diet-induced obesity and metabolic syndrome.

ACKNOWLEDGMENTS
We thank Shinji Yamamoto, Takuya Watanabe, and Kiyoshi Kataoka for preparing the coffee polyphenols.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


