Coffee and Caffeine Improve Insulin Sensitivity and Glucose Tolerance in C57BL/6J Mice Fed a High-Fat Diet

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We have previously demonstrated that coffee and caffeine ameliorated hyperglycemia in spontaneously diabetic KK-A¹ mice. This present study evaluates the antidiabetic effects of coffee and caffeine on high-fat-diet-induced impaired glucose tolerance in C57BL/6J mice. C57BL/6J mice fed a high-fat diet were given regular drinking water (control group), or a 2.5-fold-diluted coffee or caffeine solution (200 mg/L) for 17 weeks. The ingestion of coffee or caffeine improved glucose tolerance, insulin sensitivity, and hyperinsulinemia when compared with mice in the control group. The adipose tissue mRNA levels of inflammatory adipocytokines (MCP-1 and IL-6) and the liver mRNA levels of genes related to fatty acid synthesis were lower in the coffee and caffeine groups than those in the control group. These results suggest that coffee and caffeine exerted an ameliorative effect on high-fat-diet-induced impaired glucose tolerance by improving insulin sensitivity. This effect might be attributable in part to the reduction of inflammatory adipocytokine expression.

Key words: coffee; caffeine; diet-induced impaired glucose tolerance; antidiabetic effect; insulin resistance

Type 2 diabetes is a chronic disease associated with high rates of morbidity and premature mortality. Although the pathogenesis of type 2 diabetes is known to be complex, previous studies have shown that its process involves both polygenic and environmental factors.¹⁻³ The importance of the primary prevention of type 2 diabetes has been increasingly recognized in recent years, and this has stimulated interest in the role of dietary factors in the etiology of type 2 diabetes.

Coffee is one of the world’s most popular beverages. The numerous beneficial health effects of coffee consumption have recently received significant scientific attention; epidemiological studies have suggested that regularly drinking coffee prevents chronic diseases, and especially metabolic disorders such as type 2 diabetes.⁴⁻⁷ The potential to suppress hyperglycemia through a popular beverage such as coffee could be exploited as a comfortable strategy to prevent type 2 diabetes. An experimental study using a diabetic animal model would therefore be effective for evaluating the antidiabetic potency of coffee. However, few such studies have been performed, and the mechanism underlying the antidiabetic effect of coffee and its main antidiabetic compounds are not fully understood.

We have previously investigated the preventive effect of coffee on the development of hyperglycemia in KK-A¹ mice which spontaneously develop type 2 diabetes.⁸ The KK-A¹ mouse is a polygenic model for type 2 diabetes; it exhibits obesity due to hyperphagia and develops hyperglycemia at 6 weeks of age. Hyperinsulinemia, hypertriglyceridemia, and fatty liver are also apparent. Our previous results have shown that coffee and caffeine had antidiabetic effects, and suggested that coffee and caffeine could ameliorate hyperglycemia by improving the insulin resistance in KK-A¹ mice. Coffee or caffeine ingestion also reduced the inflammatory adipocytokine expression in adipose tissue and decreased fat accumulation in the liver.⁹ It was expected from these results that coffee and caffeine would exhibit antidiabetic effects in other diabetic animal models with insulin resistance.

Recent epidemiological studies have indicated that the consumption of a high-calorie diet and the ensuing obesity were two of the principal causes of type 2 diabetes.¹⁰ An obesity-induced experimental model of type 2 diabetes induced by a high-calorie diet would be useful for evaluating the antidiabetic action of coffee and caffeine. C57BL/6 mice fed a high-fat diet (HFD) have been shown to be a representative model of obesity-induced diabetes.⁸,¹⁰ HFD causes insulin resistance in the skeletal muscle, liver, and adipose tissue, and the HFD-induced increase in the serum concentration of free fatty acids (FFAs) provokes the impairment of insulin signaling in several tissues.¹¹

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Abbreviations: HFD, high-fat diet; FFA, free fatty acid; 200CA, 200 mg of caffeine/L; GTT, glucose tolerance test; ITT, insulin tolerance test; TG, triglycerides; TC, total cholesterol; WAT, white adipose tissue; BAT, brown adipose tissue; PL, phospholipids; AUC, area under the curve; SREBP-1, sterol regulatory element binding protein-1; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; PPAR-α, peroxisome proliferator activator receptor-alpha; CPT-1, carnitine palmitoyltransferase-1; IRS-1, insulin receptor substrate-1; JNK, c-jun N-terminal kinase; IKK, inhibitor of NF-κB kinase; ERK, extracellular signal-related kinase; SOCS, suppressor of cytokine signaling; JAK-STAT, Janus kinase-signal transduction and activator of transcription; DAG, diacylglycerol; AMPK, AMP-activated protein kinase

We examined in this study whether or not the ingestion of coffee and caffeine would prevent HFD-induced impaired glucose tolerance in C57BL/6J mice. The results show that the ingestion of coffee or caffeine improved insulin sensitivity and glucose tolerance. These effects suggest that the reduced mRNA levels of inflammatory adipocytokines in adipose tissue and the reduced mRNA levels of genes related to fatty acid synthesis in the liver both contributed to the antidiabetic effect of coffee or caffeine.

Materials and Methods

Chemicals. Regular canned black coffee was presented by Pokka Corporation (Aichi, Japan) and used after 2.5-fold dilution with water. The respective concentrations of caffeine, chlorogenic acid, and trigonelline in the diluted coffee used in this experiment were 250, 120, and 180 mg/L. Caffeine was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Seven-week-old male C57BL/6J mice (SLC Japan, Shizuoka, Japan) were used for this experiment. They were maintained at a controlled temperature of 23 ± 2°C and 55 ± 5% humidity with a 12-h light/dark cycle. The mice were allowed free access to water and a standard CE-2 laboratory diet (Clea Japan, Tokyo, Japan) for 5 d before the experiment was begun.

The acclimatized eight-week-old mice were assigned to three groups and respectively given water (control group, 5 mice), diluted black coffee (black coffee/water = 1:1.5; coffee group, 6 mice), or a caffeine solution (200 mg caffeine/L; 200CA group, 6 mice) for drinking. The coffee and caffeine solutions were prepared every second day, the undiluted coffee being stored at −20°C until being diluted. The mice were allowed free access to drinking water and a Quick Fat high-fat diet (Clea) for 17 weeks.

Blood samples were collected from the tail vein 2, 5, 8, 11, and 17 weeks after the start of the experiment for measuring the serum glucose concentration. The blood samples were collected at 10:00 a.m., 1 h after the food had been removed. A glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed during the experiment. The mice were killed by decapitation at the end of the experiment (between 10:00 a.m. and 12:00 p.m.), and the blood was collected. These blood samples were used to measure the serum insulin, triglyceride (TG), and total cholesterol (TC) concentrations. The liver, white adipose tissues (WAT; subcutaneous, epididymal, mesenteric, and retroperitoneal fat tissues), and interscapular brown adipose tissue (BAT) were removed and weighed. Subcutaneous fat tissue is defined as the fat pads below the root of the forefoot on the side of the body. The tissue samples were then immediately frozen in liquid nitrogen and kept at −80°C until needed. The collected blood was kept at room temperature for 15 min for coagulation. The serum was then obtained from the coagulated blood collected by centrifugation at 2000 rpm for 10 min at 4°C and then kept at −30°C prior to its use.

The animal care and experimental procedures were approved by the Animal Research Committee of Nagoya University and were conducted according to the Regulations for Animal Experiments at Nagoya University.

Analysis of the metabolic parameters. Serum glucose was measured with a Glucose C-test assay kit (Wako Pure Chemical Industries) by the glucose oxidase method. The serum triglyceride and cholesterol concentrations were respectively measured with a Triglyceride-C Test kit (Wako Pure Chemical Industries) and a Cholesterol-C Test kit (Wako Pure Chemical Industries) by the cholesterol oxidase method, and with a Glucose C-test assay kit (Wako Pure Chemical Industries) by the glucose oxidase method. A commercially available ELISA kit was used to determine the serum concentration of insulin (Morinaga Seikagaku, Kanagawa, Japan).

Glucose tolerance test (GTT). GTT was performed 3, 9, and 16 weeks after the start of the experiment. After 14 h of fasting, blood samples were collected from the tail vein (the fasting blood sample was the 0 min sample in GTT). A glucose solution (Otuka Seiyaku, Tokyo, Japan) was then intraperitoneally injected (2 g/kg of BW). Blood samples were again collected from the tail vein 30, 60, and 120 min after the glucose injection, and the serum glucose concentration was measured. The area under the curve (AUC; mg.min/L) for the glucose concentration was calculated according to the trapezoidal rule from the glucose measurements at fasting (0 min), 30, 60, and 120 min.

Insulin tolerance test (ITT). ITT was performed 15 weeks after the start of the experiment. After a 14-h fast, blood samples were collected from the tail vein (the fasting blood sample was the 0 min sample in ITT). A human insulin solution (Humulin, 0.2 units/kg of BW; Eli Lilly, Kobe, Japan) was then intraperitoneally injected. Blood samples were collected from the tail vein 30, 60, and 120 min after the injection, and the serum glucose concentration was measured.

Hepatic lipid analysis. Frozen livers were homogenized with chloroform-methanol (2:1), and the liver lipids were extracted into an organic solvent. A portion of this extract was dried, and the hepatic contents of TG, TC, and phospholipids (PL) were respectively measured by a Triglyceride E-Test, Cholesterol E-Test, and phospholipid C-Test (Wako Pure Chemical Industries). This extract was also used to measure the total lipid content according to the method of Folch, Lees, and Sloane Stanley.

RNA preparation and expression level analysis. Total RNA was extracted from frozen tissues by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), before being treated with DNase by using a Turbo DNA-free kit (Ambion, Austin, TX, USA). cDNA was synthesized by using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified by real-time PCR, using an ABI 7300 real-time PCR system with the PCR Master Mix reagent and probes (TaqMan gene expression assays; Applied Biosystems). The level of each mRNA was normalized to that of corresponding 18S rRNA.

Statistical analysis. Each result is expressed as the mean ± SEM. Phenotypic data were statistically analyzed by one-way ANOVA and the Tukey-Kramer multiple-comparison test. Values of p < 0.05 were considered statistically significant (StatView, SAS Institute, Cary, NC, USA).

Results

Effects of coffee or caffeine ingestion on blood glucose, glucose tolerance, and insulin resistance in HFD-fed C57BL/6J mice

Eight-week-old C57BL/6J mice ingested the coffee solution, caffeine solution, or water as their drinking liquid for 17 weeks. The body weight, food intake, water intake, and tissue weight are shown in Table 1. The final body weight did not differ among the control, coffee, and 200CA groups. The food intake and water intake (measured after 4, 10, and 16 weeks) were also no different among these three groups. Likewise, the liver weights of the coffee and 200CA groups did not differ from that of the control group.

C57BL/6J mice were fed in this study with a mild high-fat diet containing 13.6% of fat, the non-fasting blood glucose concentration therefore did not increase in the control group during the course of the experiment and did not differ among the three groups at any time during the experiment (Fig. 1).

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120 min, and AUC during GTT in the 200 CA group were significantly lower than the respective values in the control group (Fig. 2B and D). The blood glucose concentrations 16 weeks later after 60 and 120 min, and AUC during GTT were significantly lower in the coffee and 200 CA groups than in the control group (Fig. 2C and D).

The effects of coffee and caffeine ingestion on the insulin sensitivity were confirmed by performing ITT 15 weeks after the start of the experiment. The glucose-lowering effect of insulin was greater in the coffee and 200 CA groups than in the control group, and the blood glucose concentrations 120 min after the insulin injection were significantly lower in the coffee and 200 CA groups than in the control group (Fig. 3A). The non-fasting serum insulin concentrations at the end of the experiment were significantly lower in the coffee and 200CA groups than in the control group (Fig. 3B).

Table 1 shows the serum TG and TC concentrations, liver total lipid content, liver TG content, liver TC content, and liver PL content. The serum TG concentration was lower in the 200CA group than in the control group, although the serum TC concentration did not differ among the three groups. The mean values for the liver total lipids and TG contents in the coffee and 200CA groups tended to be lower than those in the control group. The liver TC and PL contents also did not differ among the three groups.

Changes to the adipocytokine mRNA levels in the epididymal fat tissue due to coffee and caffeine ingestion

We investigated the mechanism by which coffee and caffeine improved insulin sensitivity by measuring the epididymal fat tissue mRNA levels of adipocytokines that are known to influence insulin sensitivity. The results are shown in Fig. 4A. MCP-1, TNF-α and IL-6 are adipocytokines that cause insulin resistance in peripheral tissues. The mRNA level of MCP-1 in the 200CA group was significantly lower than that in the control group. The MCP-1 mRNA level in the coffee group tended to be lower than that in the control group, although not significantly ($p = 0.08$). The mRNA level...
of TNF-α tended to be lower in the 200CA group than in the control group, but again not significantly (p = 0.105). The mRNA levels of IL-6 in the coffee and 200CA groups were significantly lower than that in the control group. Adiponectin and leptin are adipocytokines that improve insulin sensitivity. The mRNA levels of adiponectin did not differ among these three groups, although the leptin mRNA level was significantly lower in the 200CA group than that in the control group (p = 0.15 for coffee).

**Discussion**

We have demonstrated in this study that coffee and caffeine ingestion both improved glucose tolerance and hyperinsulinemia in C57BL/6J mice fed with HFD. These effects of coffee and caffeine ingestion were not accompanied by either a reduction in food intake or any body weight loss. The result of the insulin tolerance test shows that coffee and caffeine ingestion both enhanced the insulin sensitivity of C57BL/6J mice fed with HFD. It is considered that the improved insulin resistance conferred by coffee or caffeine ingestion contributed to the suppressive effect on HFD-induced impaired glucose tolerance.
HFD consumption is the principal cause of type 2 diabetes, and the HFD-fed C57BL/6J mouse is well recognized as a model of obesity-induced diabetes.9,10) HFD has increased the FFA concentration in serum, and this elevation inhibited insulin signaling in the peripheral tissues.11) HFD has also caused hypertrophy of adipocytes which induced the production and secretion of such macrophage chemoattractants as MCP-1.14) The macrophages recruited by MCP-1 subsequently infiltrate adipose tissues, leading to a pro-inflammatory state. The infiltrating macrophages and hypertrophied adipocytes extensively secrete MCP-1 and such other adipocytokines as TNF-α and IL-6. These adipocytokines in the circulation have been reported to induce insulin resistance in peripheral tissues.15–17) TNF-α has been indicated to phosphorylate the serine/threonine residue of insulin receptor substrate-1 (IRS-1) through the activation of either c-jun N-terminal kinase (JNK), the inhibitor of NF-κB kinase β (IKKβ), or extracellular signal-related kinase (ERK).18) Phosphorylation of IRS-1 by TNF-α interferes with the subsequent insulin-stimulated tyrosine phosphorylation of IRS-1, leading to insulin resistance. IL-6 upregulates the suppressor of cytokine signaling (SOCS) expression by activating the pathway for Janus kinase-signal transduction and transcription (JAK-STAT) in the liver and adipose tissues, and inhibits insulin signaling.19,20) IL-6 expression is also induced by TNF-α.

The results of this study show that neither coffee nor caffeine ingestion affected the white adipose tissue weight (subcutaneous, epididymal, mesenteric, and retroperitoneal fat tissues). Measurement of the adipocytokine mRNA levels in adipose tissues revealed that the IL-6 mRNA levels were significantly lower in the coffee and 200CA groups than in the control group (Fig. 4A). The MCP-1 mRNA level in the 200CA group was significantly lower than that in the control group, while the MCP-1 mRNA level in the coffee group also tended to be lower than that in the control group. These results all suggest that either coffee or caffeine ingestion lowered the production of inflammatory adipocytokines and restrained adipose tissue inflammation, resulting in an amelioration of the whole-body insulin resistance. Although we plan to clarify how coffee acts on insulin signaling in the peripheral tissues, it is possible that adipose tissue is one of the targets for the direct action of both coffee and caffeine. This hypothesis is supported by the results of a previous study in which caffeine suppressed TNF-α expression in a primary culture of human adipocytes.21) Fukushima et al. have also reported that the inflammatory cytokine gene expression in adipose tissue and liver were reduced by ingesting decaffeinated or caffeine-containing instant coffee in C57BL/6J mice fed with HFD.22)

It has been elucidated that the increase of hepatic lipids caused insulin resistance in the liver.23) The hepatic accumulation of such lipid metabolites as diacylglycerol (DAG) is known to activate PKCε, this activated PKCε binding to the insulin receptor and inhibiting its tyrosine kinase activity. The activation of PKCε by lipid metabolites may thereby also interfere with the insulin action to phosphorylate tyrosine...
residues of IRS-1 and IRS-2, resulting in insulin resistance in the liver.\textsuperscript{24,25} The respective hepatic total lipid contents in this study in the control, coffee, and 200CA groups were 66.2 ± 6.4, 60.6 ± 2.3, and 60.5 ± 2.4 mg/g (Table 1), while the respective hepatic TG contents were 25.6 ± 4.1, 21.5 ± 1.6, and 20.4 ± 1.6 mg/g. Although the mean values of these contents in the coffee and 200CA groups were lower than the control values, we could not detect any significant differences. We subsequently examined alterations in the hepatic expression of genes involved in fatty acid synthesis and oxidation. Interestingly, our results show that the expression of the genes related to fatty acid synthesis (SREBP-1, FAS, and ACC) was decreased in the mice ingesting either coffee or caffeine (Fig. 4B). Expression of the FAS and ACC genes is known to be upregulated by transcription factor SREBP-1.\textsuperscript{1,33} As the hepatic SREBP-1 mRNA level was lowered by coffee or caffeine ingestion when compared to the control group, this lowering could have contributed to the reduction in FAS and ACC mRNA levels in the liver. In contrast, coffee or caffeine ingestion did not affect the hepatic expression of genes related to fatty acid oxidation (PPAR-\(\alpha\) and CPT-1). It is thus presumed that ingesting either coffee or caffeine suppresses fatty acid synthesis and led to an improvement of the fatty liver. Interestingly, Murase \textit{et al.} have reported that ingesting coffee polyphenols reduced the liver mRNA level of SREBP-1 and the liver TG content in C57BL/6J mice fed with HFD.\textsuperscript{26} The present results suggest that the amelioration of hyperinsulinemia caused a decrease in the expression of SREBP-1 genes. It has previously been revealed that insulin stimulated SREBP-1 gene expression.\textsuperscript{27} As the non-fasting serum insulin concentration was significantly lower in both the coffee and 200CA groups than in the control group (Fig. 3B), this reduction could have contributed to the decrease in SREBP-1 gene expression. It has also been reported that the expression of IRS-2 was downregulated by SREBP-1,\textsuperscript{28,29} however, the hepatic IRS-2 mRNA level in the present study was not altered by either coffee or caffeine ingestion (data not shown). We observed in our previous study that coffee or caffeine ingestion ameliorated the fatty liver in KK-\(\alpha\) mice which spontaneously developed steatohepatitis and hyperinsulinemia. It is speculated that the ameliorative effect of coffee and caffeine on fatty liver would occur in both KK-\(\alpha\) mice and C57BL/6J mice fed with HFD.

Coffee and caffeine were equally effective for HFD-induced impaired glucose tolerance in this study, so caffeine is suggested to be one of the most effective antiadipic compounds in coffee. Caffeine has been shown to have such biological functions as acting as an adenosine receptor antagonist,\textsuperscript{29} activating AMP-activated protein kinase (AMPK) in skeletal muscle,\textsuperscript{30} and protecting pancreatic beta-cells against streptozotocin toxicity.\textsuperscript{31} Further investigation is needed to determine whether or not these functions of caffeine were involved in the antiadipic effect of this compound.

Previous epidemiological studies have demonstrated that ingesting both coffee and decaffeinated coffee was correlated with a reduction in diabetes incidence.\textsuperscript{65} It has also been reported that decaffeinated coffee ingestion might improve the insulin sensitivity of the skeletal muscle in rats.\textsuperscript{321} and that coffee components other than caffeine had a beneficial effect on glucose metabolism.\textsuperscript{33–38} These results lead us to speculate that there are antiadipic compounds in coffee other than caffeine. We are currently searching for these unidentified compounds.

In conclusion, we have demonstrated that both coffee and caffeine had an antiadipic effect on HFD-fed C57BL/6J mice. The ingestion of either coffee or caffeine improved the insulin sensitivity and glucose tolerance in this mouse model. The reduction in the adipose tissue mRNA levels of inflammatory adipocytokines and in the hepatic mRNA levels of genes relating to fatty acid synthesis might have contributed to the antiadipic effect of coffee and caffeine. These actions of coffee and caffeine were similar to their actions observed in spontaneously diabetic KK-\(\alpha\) mice.\textsuperscript{31} As HFD-induced type 2 diabetes occurs frequently in humans, the present results suggest that coffee consumption would be effective as a preventive measure against human type 2 diabetes.

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